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Original Article

AMPK enhances the expression of pancreatic duodenal homeobox-1 via PPARα, but not PPARγ, in rat insulinoma cell line INS-1

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Aim: To investigate whether AMP-activated protein kinase (AMPK) regulates the expression of pancreatic duodenal homeobox-1 (PDX-1), a β-cell-specific transcription factor and whether PPARα/γ is involved in the regulation of pancreatic β-cell lines after acute stimulation.

Methods: Rat insulinoma cell line INS-1 was treated with an activator (AICAR) or inhibitor (Compound C) of AMPK as well as inhibitors of PPARs (MK886 to PPARα and BADGE to PPARγ). The mRNA levels of PDX-1, PPARα and PPARγ were measured using real-time RT-PCR, and Western blotting was used to detect the protein expression of these factors.

Results: Activation of AMPK by AICAR induced significantly increased the expression of PDX-1, and this increase was abrogated when AMPK was inactivated by Compound C. Similarly, the expression of PPARα and PPARγ was also increased by AICAR or decreased by Compound C. However AMPK activation did not increase nuclear PDX-1 protein levels when PPARα was inhibited. In contrast, AMPK activation still up-regulated PDX-1 protein levels during PPARγ inhibition. Additionally, PPARα activation induced by fenofibrate significantly enhanced nuclear PDX-1 protein expression.

Conclusion: AMPK regulates the expression of PDX-1 at both the transcriptional and protein levels, and PPARα may be acutely involved in the regulation of INS-1 cells.

Keywords: AMP-activated protein kinase; PPARα; PPARγ; PDX-1; INS-1 cells

Introduction

Adenosine 5'-monophosphate-activated protein kinase (AMPK) is an energy sensor that controls systemic glucose homeostasis by regulating metabolism in multiple tissues, including skeletal muscle, liver and pancreatic β-cells. The effects of AMPK may be related to its downstream substrates. AMPK activation increases glucose uptake concomitantly with fusion of glucose transporter 4 (GLUT4) with the plasma membrane[1, 2] and increases the expression of myocyte enhancer factor 2A (MEF2A) and MEF2D[3] in skeletal muscle. In addition, some nuclear proteins, such as p300 and hepatocyte nuclear factor 4, are downstream targets of AMPK[4]. Saleh

et al have reported[5] that pancreatic duodenal homeobox-1 (PDX-1) is one of the nuclear transcription factors that probably associates with AMPK to some extent. However, PDX-1 regulation by AMPK has not been confirmed in β cells.

The peroxisome proliferator-activated receptors (PPARs) constitute a subfamily of the nuclear receptor superfamily, which regulates gene expression in response to ligand binding. PPARα, which is expressed at low levels in pancreatic islets[6], is known to regulate fatty acid metabolism by controlling fatty acid oxidation. In skeletal muscle cells, adiponectin stimulates fatty acid oxidation by the sequential activation of AMPK, p38 MAPK and PPARα[7]. PPARγ, the most abundant PPAR in adipocytes, is known to be involved in glucose homeostasis and adipocyte proliferation[8]. In 3T3-L1 cells[9] and AMPK γ3 subunit knockout mice[10], a relationship between AMPK and PPARγ has been identified.

Our group and others independently reported that PPARα and PPARγ are able to regulate the expression of PDX-1. This

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regulation is mediated by a peroxisome proliferator-activated receptor response element (PPRE) in the promoter of PDX-1[11]. In the present study, we investigated the effect of AMPK on the expression of PDX-1, the changes of PPARα/γ and the associated mechanism.

Materials and methods

Cell culture

Rat insulinoma cell line INS-1 (at less than 40 passages) was grown in a monolayer in RPMI-1640 medium (Gibco, USA) containing 11.1 mmol/L glucose and supplemented with 10% (*v/v*) fetal bovine serum (Gibco, USA) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed every 2 to 3 d.

RNA isolation and real-time RT-PCR

Total RNA was extracted from all cells using the TRIzol (Invitrogen Corp, Carlsbad, CA) method. First-strand cDNA was generated using a commercial Takara RT kit (TaKaRa, Otsu, Shiga, Japan). The resulting cDNA was amplified by real-time RT-PCR[12] using a QuantiTect SYBR Green kit (QIA-GEN, Hilden, Germany) and an ABI 7700 Prism real-time PCR instrument and analysis software (Applied Biosystems, Foster City, CA). The following primer sequences were used in the PCR: PDX-1 sense 5'-AAACGCCACACACAAGGA-GAA-3' and antisense 5'-AGACCTGGCGGTTCACATG-3', PPARα sense 5'-TGTCACACAATGCAATCCGTTT-3' and antisense 5'-TTCAGGTAGGCTTCGTGGATTC-3', and PPARγ sense 5'-TGTGGACCTCTCTGTGATG-3' and antisense 5'-CATTGGGTCAGCTCTTGTGA-3'. All quantifications were performed with rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH, primer sequence: sense 5'-TGGTGGACCT-CATGGCCTAC-3' and antisense 5'-CAGCAACTGAGGGC-CTCTCT-3') as an internal standard. The PCR was performed with cycles at 95 °C for 15 s, 60 °C for 31 s and 72 °C for 31 s. The relative quantification of gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method[13], and the results were expressed as the extent of change with respect to control values.

Whole cell protein extraction and Western blotting

The treated cells were suspended in lysis buffer (RIPA, Shenneng Bo Cai, China). Protein concentrations of the extracts were determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). For each sample, 60 μg of protein was separated by 10% SDS-PAGE and electroblotted onto nitrocellulose membranes. The membranes were blocked with 5% (*w/v*) non-fat milk in TBST (10 mmol/L Tris, 150 mmol/L NaCl and 0.1% *v/v* Tween 20) and then incubated with a specific primary antibody for phosphorylated AMPKα subunit (P-AMPKα; Cell Signaling, Danvers, MA, USA) at 4 °C. After incubation with the corresponding secondary antibody, immune complexes were detected using an enhanced chemiluminescence (ECL) method (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). The same membrane was incubated again with a specific primary antibody for β-actin (Cell Signaling, USA).

Nuclear protein preparation and Western blotting

Nuclear proteins were extracted according to the instructions of the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA). After protein quantification, the resulting supernatants were fractionated by 12% SDS-PAGE and electroblotted following the method described above. The immunoreactive bands were quantified using an AlphaImager 2200 (Alpha Innotech, USA). The PDX-1 antibody was purchased from CHEMICON (USA).

Immunoprecipitation

Nuclear proteins were prepared as described in the Western blotting protocol above, and protein G agarose beads (Upstate, Lake Placid, NY, USA) were used to precipitate protein complexes following the manufacturer's instructions. The antibody for precipitation was a polyclonal PPARα/γ primary antibody (Santa Cruz), and the antibody for detection was a monoclonal PPARα/γ antibody (Abcam). ECL was used for visualization as described above.

Statistical analysis

All data are expressed as means±SD. Statistical analysis was performed by two-tailed unpaired Student's *t*-tests or by one-way analysis of variance (ANOVA) followed by a *post hoc* Turkey's multiple comparison test. Values of $P<0.05$ were considered to be statistically significant.

Results

AICAR enhances AMPK activity in INS-1 cells

We first confirmed that the AMPK agonist AICAR has an activating effect in our experimental cell models. INS-1 cells were treated with or without 0.5 mmol/L AICAR in the presence or absence of 10 μmol/L Compound C (an AMPK antagonist) for 8 h. Compared to the control group, AICAR enhanced the abundance of P-AMPKα by 2.27-fold ($P<0.05$; Figure 1), whereas AICAR plus Compound C co-treatment resulted in a 71.67% decrease in the abundance of P-AMPKα ($P<0.05$) relative to the AICAR treatment alone group. However, there was no significant difference in P-AMPKα levels between the control group and the group with Compound C treatment alone ($P>0.05$). Consistent with the characterization of Compound C, it only blocked AMPK activity induced by AICAR or other AMPK activators and showed no effect when used alone. These results indicate that AICAR has an activating effect on AMPK in INS-1 cells, which has been previously reported by Kim *et al*[14].

AMPK activation increases the expression of PDX-1 in INS-1 cells

To investigate the effect of AMPK on PDX-1, cells were treated with or without 0.5 mmol/L AICAR in the presence or absence of 10 μmol/L Compound C for 8 h, and changes in PDX-1 were observed at both the transcriptional and nuclear protein levels. As shown in Figure 2A, compared to the control group, AMPK activation increased PDX-1 mRNA expression by 2.38-fold ($P<0.05$), and when co-treated with AICAR plus
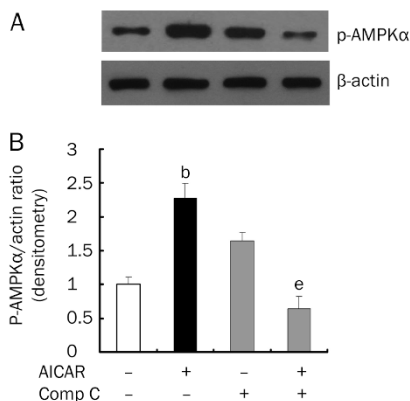
A

B

Figure 1. AICAR stimulates the activity of AMPKα. INS-1 cells treated with or without 0.5 mmol/L AICAR in the presence or absence of 10 μmol/L Compound C (Comp C) for 8 h were washed with phosphate-buffered saline and were lysed. For each sample, 60 μg protein was used for Western blotting. (A) A representative blot is shown. (B) Densitometric results from six independent Western blotting experiments are shown. Data were calculated using β-actin as an internal standard and were normalized by setting the control as 1 (mean±SD). [b]*P*<0.05 compared to the untreated control group; [e]*P*<0.05 compared to the group treated with AICAR alone.

Compound C, the expression was reduced by 34.73% (*P*<0.05) relative to the group treated with AICAR alone.

To extend the above mRNA results, PDX-1 protein levels were measured under the same conditions. As a nuclear transcription factor, PDX-1 only has activity in the nucleus. This led us to assess PDX-1 levels both in the nucleus and the cytoplasm. As shown in Figure 2B, AMPK activation induced by AICAR significantly up-regulated nuclear PDX-1 protein levels relative to the control group, and this enhancement was restored to the normal level by co-treatment with Compound C. However, as shown in Figure 2C, the cytoplasmic

PDX-1 protein expression in the AICAR group was noticeably higher than that of the control group, both in the presence and absence of Compound C. We concluded that the expression of PDX-1 is affected by AMPK activity.

AMPK activation up-regulates PPARα and PPARγ expression in INS-1 cells

Given that AMPK activity alters the expression of PDX-1, we next wondered whether the expression of PPARα and PPARγ would change as well. We detected transcriptional induction of PPARα and PPARγ in response to AMPK activation. As shown in Figure 3A and 3B, AICAR-induced AMPK activation increased the mRNA levels of PPARα and PPARγ by 2.25-fold (*P*<0.05) and 2.89-fold (*P*<0.05), respectively, compared to the control group. When Compound C was added, the mRNA levels of both PPARα and PPARγ were reduced by 61.10% (*P*<0.05) and 34.95% (*P*>0.05), respectively, relative to the group treated with AICAR alone.

Because PPARα and PPARγ are nuclear proteins, they need to be translocated into nuclei before they can function. To reconfirm changes in nuclear PPARα/γ protein in AICAR-treated cells, PPARα/γ was immunoprecipitated. As shown in Figure 3C and 3D, AICAR treatment increased nuclear PPARα protein levels by 1.36-fold (*P*<0.05) and nuclear PPARγ by 1.78-fold (*P*<0.05) relative to the control, and co-treatment with Compound C significantly reduced these inductions (*P*< 0.05). These results indicate that AMPK regulates the expression of PPARα and PPARγ in addition to that of PDX-1.

AMPK activation up-regulates nuclear PDX-1 via PPARα and not PPARγ

These findings prompted us to further investigate the specific mechanism of the regulation of PDX-1 by AMPK. We speculated that AMPK could regulate the expression of PDX-1 either directly or indirectly, and indirect regulation would involve other factors between AMPK and PDX-1. On the other hand,
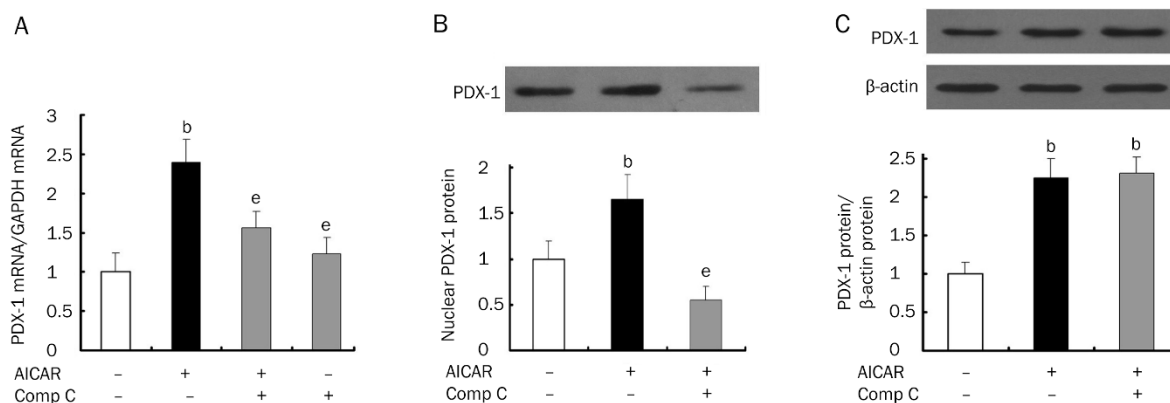
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Figure 2. AMPK activation increases the expression of PDX-1. INS-1 cells were treated with or without 0.5 mmol/l AICAR in the presence or absence of 10 μmol/L Compound C (Comp C) for 8 h, and were then harvested to monitor mRNA levels of PDX-1 (A) by real-time RT-PCR. Data were calculated using GAPDH as an internal standard and were normalized by setting the control as 1 (mean±SD, *n*=6). PDX-1 protein from nuclear (B) and cytoplasmic (C) fractions were detected by Western blotting. Densitometric results from six independent Western blotting experiments are shown. Data were calculated using β-actin as an internal standard and were normalized by setting the control as 1 (mean±SD, *n*=6). [b]*P*<0.05 compared to the untreated control group; [e]*P*<0.05 compared to the group treated with AICAR alone.
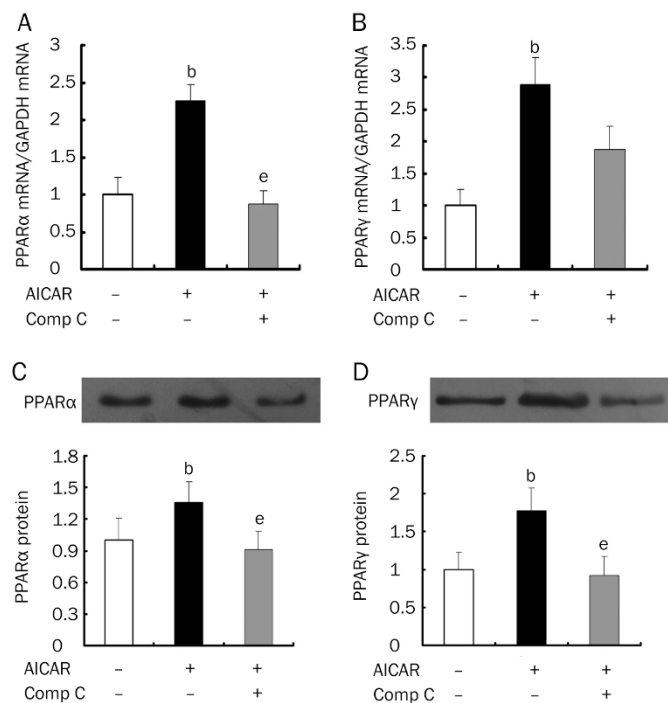
Figure 3. AICAR up-regulates PPARα and PPARγ expression. INS-1 cells were treated with or without 0.5 mmol/L AICAR, in the presence or absence of 10 μmol/L Compound C (Comp C) for 8 h. They were then harvested to monitor mRNA levels of PPARα (A) and PPARγ (B) by real-time RT-PCR. Results from six independent experiments are shown. Data were calculated using GAPDH as an internal standard and were normalized by setting the control as 1 (mean±SD). Nuclear protein fractions were prepared to detect PPARα (C) and PPARγ (D) by immunoprecipitation. Densitometric results from six independent Western blotting experiments are shown. Data were normalized by setting the control as 1 (mean±SD). [b]$P<0.05$ compared to the untreated control group; [e]$P<0.05$ compared to the group treated with AICAR alone.
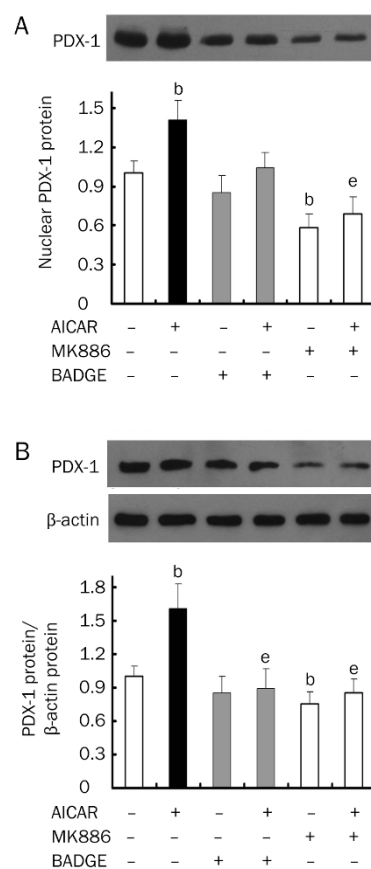
Figure 4. Effects of MK886 and BADGE on the expression of PDX-1. INS-1 cells were pre-incubated with 5 μmol/L MK 886 or 50 μmol/L BADGE for 16 h and in the presence or absence of 0.5 mmol/L AICAR for 8 h. PDX-1 protein from nuclear (A) and cytoplasmic (B) fractions was detected by Western blotting. Densitometric results from six independent Western blotting experiments are shown. Data were calculated using β-actin as an internal standard and were normalized by setting the control as 1 (mean±SD, n=6). [b]$P<0.05$ compared to the untreated control group; [e]$P<0.05$ compared to the group treated with AICAR alone.

the regulation may be related to PPARα and PPARγ because some reports have shown that there is a PPRE in the promoter of PDX-1.

To examine the relationships between PDX-1 and PPARα/γ, we used the PPARα inhibitor MK886 and the PPARγ inhibitor BADGE. We incubated cells with or without 5 μmol/L MK886 or 50 μmol/L BADGE for 16 h prior to incubation with 0.5 mmol/L AICAR for 8 h, and PDX-1 from the nuclear and cytoplasmic fractions was then detected by Western blotting. As shown in the cytoplasmic protein results in Figure 4B, compared to the increased PDX-1 expression in the group treated with AICAR alone, PDX-1 expression was inhibited by the addition of either MK886 or BADGE (*P*<0.05). Importantly, as shown in the nuclear protein results in Figure 4A, nuclear PDX-1 protein levels were significantly reduced in the cells treated with both AICAR and MK886 compared to cells treated with AICAR alone (*P*<0.05), but there were no significant changes with AICAR and BADGE co-treatment (*P*>0.05). From these results, we concluded that once AMPK is activated, the effect of P-AMPK on PDX-1 expression is mediated,

at least in part, by PPARα, not PPARγ.

Fenofibrate up-regulates nuclear protein expression of PPARα and PDX-1

To confirm the relationship between PDX-1 and PPARα, we used the PPARα agonist fenofibrate. We incubated cells with or without 5 μmol/L fenofibrate or 5 μmol/L MK886 for 8 h, and then nuclear PPARα and PDX-1 from nuclear and cytoplasmic fractions was detected by immunoprecipitation and Western blotting. As shown in Figures 5A and 5B, fenofibrate increased nuclear protein levels of PPARα and PDX-1 by 1.82-fold (*P*<0.05) and 1.67-fold (*P*<0.05), respectively, compared to the control group. However, MK886 reduced nuclear protein levels of both PPARα and PDX-1 by 79.67% (*P*<0.05) and 86.06% (*P*<0.05), respectively, relative to the control group. In Figure 5C, cytoplasmic protein levels of PDX-1 in the fenofibrate group were higher than in the control group, but there were no significant differences (*P*>0.05). However, once
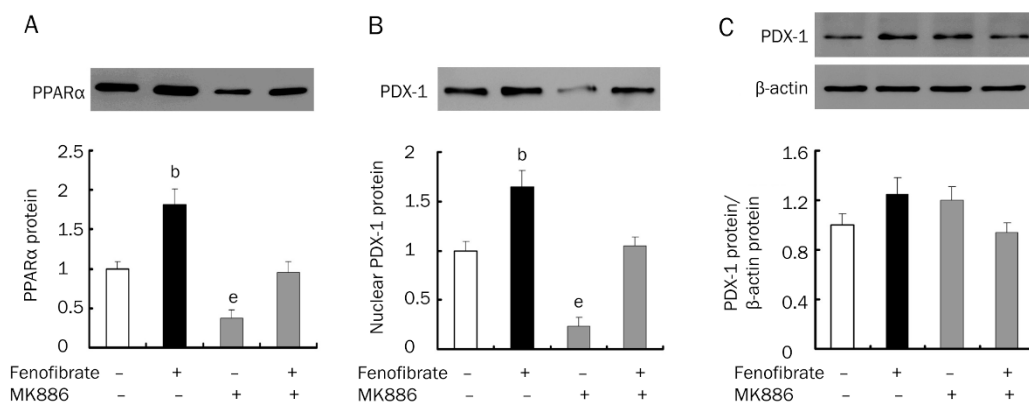
Figure 5. Effects of fenofibrate on the expression of PDX-1 and PPARα. INS-1 cells were treated with or without 5 µmol/L fenofibrate in the presence or absence of 5 µmol/L MK886 for 8 h. Nuclear PPARα protein (A) was detected by immunoprecipitation, and PDX-1 protein from nuclear (B) and cytoplasmic (C) fractions was detected by Western blotting. Densitometric results from six independent Western blotting experiments are shown. [b]$P<0.05$ compared to the untreated control group; [e]$P<0.05$ compared to the group treated with fenofibrate alone.

PPARα was activated by fenofibrate, nuclear PDX-1 expression was enhanced.

Discussion

In this study, AICAR-induced AMPK activation up-regulated the expression of PDX-1 both at the transcriptional and protein levels in INS-1 cell lines. In addition, we found that AICAR could affect the mRNA and nuclear protein levels of PPARα and PPARγ under specific physiological conditions. We also found that the increase in PDX-1 induced by AICAR and fenofibrate could be reversed by PPARα inhibition. Collectively, these findings suggest that PPARα might be involved in the regulation of AMPK on PDX-1 to some extent.

PDX-1 plays a crucial role in both pancreas development and maintenance of β-cell function. Targeted disruption of the PDX-1 gene in β-cells of mice leads to overt diabetes and decreased insulin expression and secretion. In humans, mutations in the PDX-1 gene have been linked to diabetes. Hence, the identification of molecular mechanisms regulating the transcriptional activity of this key transcription factor is of great importance[15].

Until now, there have been few studies regarding the relationship between AMPK and PDX-1. When the pancreatic β-cell line INS-1E was exposed to palmitate, glucose-stimulated insulin secretion was impaired and both AMPK and PDX-1 were down-regulated[16]. A PPRE sequence has been identified in the promoter of PDX-1[11, 17]; thus, PPARs may have the ability to regulate the transcriptional activity of PDX-1. Activation of AMPK by AICAR up-regulates mRNA expression of PPARα in skeletal muscle[18], and specific knock-down of the catalytic AMPK-subunit AMPKα2 using RNAi suppressed the expression of PPARα in INS-1E cells and in rat islets[19]. The induction of PPARα and the increment of PDX-1 expression as observed in the current study are consistent with data demonstrating that PPARα enhancement induced by the PPARα agonist fenofibrate could lead to an increase in PDX-1mRNA and nuclear protein, as well as DNA-binding activity

of PDX-1 to the insulin promoter. Additionally, fenofibrate induced overexpression of downstream targets of PDX-1, such as insulin and GLUT2[17]. Similarly, in islets cultured with palmitate for 8 h, both PPARα and PDX-1 mRNA expression increased[20]. Our results indicate that AMPK may regulate the expression of PDX-1 in INS-1 cells, where AMPK activation causes up-regulation of PDX-1. This conclusion is based on observations that AMPK activation enhanced the PDX-1 mRNA and nuclear protein expression. Further support for this relationship is gained from the results of experiments using the PPARα antagonist MK886, in which we detected an inhibition of these cellular events. A similar β-cell dysfunction has been reported in mice with a PPARα gene knockout and in pancreatic β-cells unable to express PDX-1[21].

If activation of PPARα increases PDX-1 expression, PPARα inhibition might be expected to reduce the expression of PDX-1. Our study validates this prediction. MK886 is an effective PPARα inhibitor and has functional effects. MK886 used at 10–20 µmol/L inhibited PPARα activation induced by Wy14643 by approximately 80% in monkey kidney fibroblast CV-1 cells, mouse keratinocyte 308 cells and human lung adenocarcinoma A549 cells[22]. In addition, PPARα-mediated Acyl-CoA oxidase levels are very low in keratinocytes exposed to MK886. There are effects of MK886 on PPARβ and PPARγ, but only minimal inhibitory effects were observed. Therefore, in our study, we observed that nuclear PPARα protein levels were reduced when cells were treated with MK886. Additionally, nuclear PDX-1 levels were reduced significantly when AICAR and MK886 were used together compared to the group treated with AICAR alone. We therefore consider the effect of MK886 to be mainly attributable to PPARα inhibition.

In 3T3-L1 cells incubated in a standard adipogenic medium, the activation of AMPK by AICAR dramatically inhibited the expression of PPARγ, and inhibition of AMPK by Compound C enhanced the expression of PPARγ[9]. In sharp contrast, the expression of PPARγ was enhanced in skeletal muscles of the skeletal muscle-specific transgenic mice (named Tg-Prkag3[225Q]

mice) during fed and fasted conditions, and this was restored in Prkag3$^{-/-}$ mice (AMPKγ3 subunit knockout mice)[10]. The current study showed that AMPK activation up-regulated the expression of PPARγ in INS-1 cells. The main function of β-cells is to detect glucose changes and secrete insulin appropriately, but adipocytes from peripheral tissues are very different from β-cells; the cell type-specific differences are a possible reason for the inconsistent results.

The results regarding the relationship between PPARγ and PDX-1 contradict our initial hypothesis. PPARγ activation is known to inhibit proliferation and have pro-differentiation effects in many tissues. When isolated islets were incubated with the PPARγ agonist troglitazone, islet proliferation was reduced and PDX-1 expression increased[23]. In the current study, we speculated that PPARγ regulates the expression of PDX-1. However, nuclear PDX-1 protein levels were not obviously affected by the combination of AICAR and BADGE. We know BADGE is a synthetic antagonist for PPARγ. This compound has no apparent ability to activate the transcriptional activity of PPARγ; however, BADGE can antagonize the ability of agonist ligands such as rosiglitazone to activate the transcriptional activity of PPARγ[24, 25]. It is also possible that PPARγ is an upstream factor of PDX-1 because there may have been problems with the concentration of BADGE and the exposure time used in our study. Although other transcription factors are involved in the regulation of AMPK on PDX-1, PPARα might be a player in the regulation of PDX-1 transcription.

Acknowledgements

Author contribution

Hua GUO performed the research, analyzed the data, and wrote the paper; Shui SUN performed the research, analyzed the data, and wrote the paper; Xu ZHANG and Xiu-juan ZHANG performed the research; Ling GAO designed the study, performed the research, analyzed the data and wrote the paper; Jia-jun ZHAO designed the study, analyzed the data, and wrote the manuscript.

Abbreviations

AMPK, adenosine 5'-monophosphate-activated protein kinase; PDX-1, pancreatic duodenal homeobox-1; PPARα, peroxisome proliferator-activated receptor-alpha; PPARγ, peroxisome proliferator-activated receptor-gamma; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; BADGE, bisphenol A diglycidyl ether

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