AICAR enhances insulin signaling via downregulation of miR-29

Journal:	<i>Canadian Journal of Physiology and Pharmacology</i>
Manuscript ID	cjpp-2015-0159.R1
Manuscript Type:	Article
Date Submitted by the Author:	21-Jul-2015
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Keyword:	AMP-activated protein kinase, insulin signaling pathway, microRNA-29, phosphoinositide 3-kinase, p85α

AICAR enhances insulin signaling via downregulation of miR-29

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Abstract

As an activator of AMPK, AICAR's effect on insulin signaling has not been investigated extensively. PI3K/Akt is critical nodes involved in insulin signaling pathway. We observed that concomitant with the activation of AMPK by AICAR, the protein level of PI3K p85 α and the insulin-induced phosphorylation of Akt were enhanced in mouse primary hepatocytes. Previously, we identified a group of AMPK-regulated miRNAs in hepatocytes. It is not clear that whether miRNAs are related to the regulation of insulin signaling by AMPK. Here, we confirmed the negative regulation of miR-29 family members by AICAR treatment in mouse primary hepatocytes. Our results indicated that p85 α is a direct target of miR-29 and is negatively regulated by miR-29b in hepatocytes. In agreement with the findings *in vitro*, we found that the expression of miR-29 and the protein levels of p85 α were inversely correlated in the liver of fasted mice. Overexpression of miR-29b reduced the insulin-induced phosphorylation of Akt in hepatocytes, suggesting that miR-29 could serve as a negative regulator of insulin signaling. Furthermore, we found that overexpression of miR-29 could attenuate the effect of AICAR on p85 α expression. Taken together, our results indicated that activation of AMPK might enhance insulin signaling via downregulation of miR-29.

Keywords: AMP-activated protein kinase, insulin signaling pathway, microRNA-29, phosphoinositide 3-kinase, p85 α

Introduction

The activity of AMP-activated protein kinase (AMPK) is suppressed in disorders closely related to insulin resistance (Luo et al. 2005). 5-Aminoimidazole-4-carboxamide 1- β -D-ribofuranoside (AICAR), an AMPK activator, has been suggested in the regulation of hepatic glucose and lipid metabolism (Ruderman and Saha 2006; Zhou et al. 2001). Meanwhile, it has also been reported that the association of p85 α subunit of phosphoinositide 3-kinase (PI3K) with IRS-1 and subsequent Akt phosphorylation were reduced in the liver of high fat-fructose diet-feeding mice due to AMPK activation (Yogalakshmi et al. 2014). However, whether and how AMPK signaling affects hepatic insulin signaling is largely unknown.

There are several reports discussing the roles of miRNAs in multiple metabolic processes, in which make it well acknowledged that miRNAs play great roles in the development and progression of metabolic diseases (Esau et al. 2006; Wang et al. 2013). While quite a few investigations (Godlewski et al. 2010a; Godlewski et al. 2010b) have reduced groups of miRNAs which show altered expression profiles under AMPK signaling pathway. Our laboratory carried out miRNA microarray study recently (Liu et al. 2013) has shown decreased levels of miR-29 family members in mouse primary hepatocytes treated with AICAR. Besides He et al. informed its role in diabetes (He et al. 2007), miR-29 is related to several physiopathology process including fibrosis, sclerosis, apoptosis, myogenesis, and tumorigenesis (Kapinas et al. 2009; Maurer et al. 2010; Mott et al. 2007; Wang et al. 2008). However, whether miR-29 is involved in the crosstalk between AMPK signaling and insulin signaling pathway is not clear.

One of the critical nodes of insulin signaling pathway is PI3K, which is activated by binding of the p85 α regulatory subunit to specific sites on IRS1/IRS2 (Harrington et al. 2005). Recently, the role of miRNAs in the regulation of insulin signaling has been extensively studied (Karolina et al. 2011; Wang et al. 2014). For example, miR-378 inhibits hepatic insulin signaling through targeting p110 α , a catalytic subunit of PI3K (Liu et al. 2014). It has been shown that miR-29 is able to enhance the gluconeogenesis through targeting hepatic p85 α , and the expression of phosphoenolpyruvate carboxykinase (PEPCK) increased (Pandey et al. 2011). However, whether miR-29 could affect insulin-induced signaling transduction has not been investigated.

In this study, we observed that activation of AMPK by AICAR could increase insulin-induced phosphorylation of Akt as well as the protein levels of p85 α in mouse primary hepatocytes, suggesting that activation of AMPK signaling is able to amplify insulin signaling. Since we found that miR-29 levels were decreased in the AICAR-treated hepatocytes, we hypothesized that miR-29 might be involved in the regulation of insulin signaling by AMPK. Indeed, we found that miR-29 directly targets p85 and inhibits insulin-induced phosphorylation of Akt in hepatocytes. Thus this current study is a remarkable addition to the present knowledge of miRNAs in AMPK related insulin signaling pathway.

Materials and Methods

Animal experiments

Wild-type C57BL/6 mice (eight, male), purchased from Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China), were randomly divided into two groups with 4 mice per group, which were kept under standard conditions with 12 hours light/dark cycles and access to food and water ad libitum before 8 weeks old. One group of mice was fasted for 14 h, and livers from both groups were immediately removed and snap-frozen in liquid nitrogen. All animal care and procedures were performed according to the instructions of 'The Animal Care and Welfare Committee' of the Peking Union Medical College (Beijing, China).

Cell culture and transfection

Primary mouse hepatocytes were isolated and cultured as previously described (Liu et al. 2013). Mouse Hepa1-6 hepatocytes were cultured as described (Djian et al. 1985). The miR-29 gain-of-function study was performed using miR-29b mimics (100 nM) and its negative control (100 nM) on the C57BL/6 mouse primary hepatocytes. The miR-29b mimics sequence is 5'- UAGCACCAUUUGAAAUCAGUGUU -3'. Hepatocytes were transfected using lipofectamine™ 2000 reagent (Invitrogen, USA) in DMEM (Gibco, USA), according to the manufacturer's instructions. The relative level of miR-29b in transfected hepatocytes was detected by qRT-PCR.

Real-time quantitative PCR analysis

Real-time quantitative PCR analysis

Total RNA extracted from cells was using Trizol reagent (Invitrogen, CA) and miRNeasy

mini kit (QIAGEN) according to manufacturer's instructions. RNA quality and quantity was measured using nanodrop spectrophotometer (ND-1000, Nanodrop Technologies). RNA integrity was determined by gel electrophoresis. The samples were reverse-transcribed to cDNA using the reverse transcription kit (TaKaRa, Japan). The expression of mRNA was quantified with real-time PCR was carried out using SYBR Green qPCR mix. The sense and antisense PCR primers used, respectively, were as follows: for p85 α , 5'-GATTTTAATCTCAACGGAATGG-3', 5'-CTAGGTGTAAGAGTGTAATCGC-3'; peroxisome proliferator-activated receptor- γ coactivator-1 α (Pgc-1 α), 5'-ATACCGCAAAGAGCACGAGAAG-3', 5'-CTCAAGAGCAGCGAAAGCGTCACAG-3'; for Pepck, 5'-GTGCTGGAGTGGATGTTCGG-3', 5'-CTGGCTGATTCTCTGTTTCAGG-3'; for glucose-6-phosphase (G6pase), 5'-ACTGTGGGCATCAATCTCCTC-3', 5'-CGGGACAGACAGACGTTCAGC-3'; and for β -actin, 5'-TGGAATCCTGTGGCATCCATGAAAC -3', 5'-TAAACGCAGCTCAGTAACAGTCCG -3'. miR-29 family's relative expression was calculated using the expression of U6 small nuclear RNA as the reference. The sequence-specific forward primers for mature miR-29 and U6 internal control were as follows: for miR-29a, 5'- UAGCACCAUCUGAAAUCGGUUA -3'; for miR-29b, 5'-UAGCACCAUUUGAAAUCAGUGUU -3'; for miR-29c, 5'-UAGCACCAUUUGAAAUCGGUUA -3'; and for U6, 5'-CTCGCTTCGGCAGCACA-3'.

Western blotting analysis

Primary mouse hepatocytes were treated with AICAR or miR-29b mimics. Cultured hepatocytes were then harvested and extracted in RIPA lysis buffer. The protein samples were separated by SDS-PAGE gels and transferred to nitrocellulose membrane. The membranes were washed with Tris-buffered saline supplemented with 0.05% NP-40 and 5% nonfat dry milk for 30 min at 37°C. Immunoblotting was performed following standard procedures, and the signals were detected using chemiluminescence reagents (Syngene). Primary antibodies were directed against: p-AMPK, p-Akt, p85 α , β -actin and tubulin (all from Santa Cruz).

Statistical analysis

Results were presented as mean $\pm$ standard deviation. Statistical analyses were assessed using *T*-test between two groups and one-way ANOVA with multiple groups. All analyses were performed with SPSS 19.0 (SPSS Inc., USA), and a value of $P < 0.05$ was considered to indicate statistical significance.

Results

Effects of AICAR on insulin signaling

AICAR is a specific activator of AMPK. The phosphorylation of AMPK increased significantly after AICAR treatment (Fig. 1A). We first investigated the effect of AICAR on insulin signaling transduction in C57BL/6 mouse primary hepatocytes. As shown in Fig. 1B, the insulin-induced phosphorylation of S473 on Akt in absence or presence of

AICAR (0.5mM, 2h) was examined in primary hepatocytes. Interestingly, we observed that AICAR augmented the insulin-stimulated phosphorylation of Akt at S473 by more than 2-fold (Fig.1B), indicating that activation of AMPK signaling might enhance insulin signaling in liver cells.

p85 α is the regulatory subunit of PI3K, which is important in insulin signaling pathway and an upstream protein of Akt. To evaluate whether AICAR treatment has any effects on p85 α expression, C57BL/6 mouse primary hepatocytes were treated with AICAR followed by insulin administration, and the p85 α protein level were examined. As shown in Fig.1C, AICAR treatment caused an increase of p85 α protein levels both in the presence and absence of insulin. These results suggested that activation of AMPK signaling might be able to enhance insulin signaling pathway by elevating the protein expression levels of p85 α .

Effects of AICAR on miR-29 family in mouse primary hepatocytes

Recently our laboratory carried out a miRNA microarray study in control versus C57BL/6 mouse primary hepatocytes (Liu et al. 2013) showed decreased levels of all miR-29 family members in AICAR-treated hepatocytes. To verify this result, real time PCR was performed to detect the expression of miR-29a, miR-29b and miR-29c in C57BL/6 mouse primary hepatocytes after AICAR treatment. AICAR concentrations 0.2 mM and 0.4 mM were chosen to the concentrations described (Cacicedo et al. 2004) to examine whether the treatment of AICAR influenced the expression of miR-29a, miR-29b and miR-29c. As shown in Fig.2, dibutyryl-cAMP (Bt₂-cAMP) treatment

increased the expression of all three miR-29 family members significantly in hepatocytes. Following AICAR treatment, the expression of miR-29a, miR-29b and miR-29c were all decreased in a dose-dependent manner, suggesting that all miR-29 family members might be involved in AMPK signaling pathway (Fig.2).

miR-29 targets p85 α regulated insulin signaling in C57BL/6 mouse primary hepatocytes

PI3K p85 α subunit had been reported as a target of miR-29 in a hepatic cell line (Park et al. 2009). However, whether miR-29 could affect the insulin-induced phosphorylation of Akt was not clear, which requires further study. In this study, we first determined whether miR-29 overexpression could decrease the protein level of p85 α in primary hepatocytes. Since the three homologs of miR-29 family have the same 'seed sequence' and the effects of AICAR on miR-29b were more evident, miR-29b was chosen to contrive miRNA mimics. As shown in Fig. 3B, the real time PCR results indicated that the transfection of miR-29b mimics effectively elevated the expression of miR-29b in primary hepatocytes ($P < 0.01$). In agreement with the previous report, overexpression of miR-29b resulted in a decrease in both mRNA and protein levels of p85 α ($P < 0.05$) (Fig. 3C and D).

To evaluated the downstream of PI3K in insulin signaling pathway, we then detected the gluconeogenesis related gene Pgc-1 α , Pepck and G6Pase expression after miR-29b mimics treatment. The real time PCR results indicated that the expression of Pgc-1 α , Pepck and G6Pase were elevated significantly after the transfection of miR-29b mimics in primary hepatocytes ($P < 0.01$) (Fig. 3E).

The expression of miR-29 and p85 α in the liver of fasted C57BL/6 mice

Since our results suggested that p85 α is a direct target of miR-29, we then examined whether the expression levels of miR-29 family members and p85 α protein levels are conversely correlated in the liver of mice under different feeding conditions. First of all, we compared the abundance of each miR-29 family members in the liver of C57BL/6 mice and primary hepatocytes. As shown in Fig. 4A, the expression of miR-29a was slightly higher than that of miR-29b or miR-29c (Fig. 4A). Interestingly, we found that the expression levels of all three miR-29 family members increased significantly in the liver of mice during fasting (Fig. 4B). In contrast, both of mRNA and protein levels of p85 α decreased significantly in the liver of mice upon fasting. These *in vivo* results further supported the notion that p85 α is a target of miR-29. Insulin receptor- β increased, and its downstream the phosphorylation of insulin-receptor, substrate IRS1, Akt reduced in skeletal muscle biopsies of volunteer subjects after fasting (Wijngaarden et al. 2013). Our data also indicated that the elevation of miR-29 expression and the repressed p85 α protein expression might facilitate the inhibition of insulin signaling in fasted status.

Effects of miR-29b on insulin signaling

We then assessed the effect of miR-29b mimics on insulin-induced phosphorylation of S473 on Akt in C57BL/6 mouse primary hepatocytes. As shown in Fig. 5A, the miR-29b mimics incubation markedly inhibited insulin-induced Akt phosphorylation ($P < 0.01$). Similar results were obtained in Hepa1-6 hepatocytes. These results suggested that miR-29b was able to inhibit insulin signaling.

MiR-29 diminishes the effect of AICAR on the expression of p85 α

Based on our findings, we hypothesized that the repression of miR-29 by AICAR, which leads to the derepression of p85 α , might be involved in the augment of insulin signaling by AICAR. To further test this hypothesis, we investigated whether overexpression of miR-29b could antagonize the effect of AICAR on p85 α . As shown in Fig. 6, AICAR treatment enhanced the protein expression level of p85 α , which could be repressed by overexpression of miR-29b. These results further supported our hypothesis that miR-29b might mediate the effects of AICAR on insulin signaling.

Discussion

In previously study, we found that the expression levels of all of the miR-29 family members were significantly decreased in AICAR treated C57BL/6 mouse primary hepatocytes compared with normal control (Liu et al. 2013), implying that miR-29 family members might be involved in hepatic AMPK signaling pathway. In mice, either the miR-29b-1/miR-29a cluster or the miR-29b-2/miR29c cluster encodes the mice's three miR-29 paralogs, miR-29a, miR-29b and miR-29c (Hwang et al., 2007). In this research, the expression of miR-29a, miR-29b and miR-29c were all decreased in a dose-dependent manner, suggesting that all the miR-29 family members might be involved in AMPK signaling pathway.

Complex relationship exists between the AMPK and insulin signaling pathways. It was reported that AMPK regulate the activation IRS-1 (Tzatsos and Tschlis 2007) and Akt

(Levine et al. 2007), while AMPK activation is negatively impacted by insulin and Akt in liver (Hahn-Windgassen et al. 2005; Qiao et al. 2002). We observed that AICAR augmented the insulin-stimulated phosphorylation of Akt at S473 by more than 2-fold (Fig.1A), indicating that activation of AMPK signaling might enhance insulin signaling in liver cells, which was the same with previous research (Tao et al. 2010). p85 α is the regulatory subunit of PI3K, which is important in insulin signaling pathway and a direct upstream protein of Akt. AICAR treatment caused an increase of p85 α protein levels both in the presence and absence of insulin, which suggested that activation of AMPK signaling might be able to enhance insulin signaling pathway by elevating the protein expression level of p85 α .

He et al. found that the increase in miR-29 level caused insulin resistance, resemble that of incubation with high glucose and insulin. And high glucose and insulin may induced the expression of miR-29a and miR-29b (He et al. 2007). These results suggested that miR-29 family were related to glucose metabolism. We searched miR-29 family's targets with TargetScan v5.2 (<http://www.targetscan.org>) that the p85 α regulatory subunit of PI3K binding sites for miR-29 at positions of its 3'UTR 331~337 (Fig. 3A). And it is conserved across species. It was validated in Hela cells by Park et al. that led to negative regulation of the tumor suppressor protein, p53 (Park et al. 2009), and in HepG2 cells by Panday et al. that prevented insulin stimulated Akt phosphorylation even in the presence of unaltered total Akt levels. We speculated that AMPK might increase insulin signaling pathway by miR-29 targeting p85 α . In this study, we first determined whether miR-29 overexpression could decrease the protein level of p85 α in primary hepatocytes. Since the

three homolog of miR-29 family have the same 'seed sequence' and the effects of AICAR on miR-29b was more evident, miR-29b was chosen to contrive miRNA mimics.

MiR-29b mimics mediated overexpression of miR-29b in mouse primary hepatocytes could largely decrease both mRNA and protein levels of p85 α . Liang et al. found that overexpression of miR-29a-c in hepatocytes decreased the protein levels of Pgc-1 α and G6Pase, and hence reduced hepatocytes and hepatic glucose production (Liang et al. 2013). And our results showed that the gluconeogenesis related gene Pgc-1 α , Pepck and G6Pase were increased after miR-29b mimics treatment, which was consistent with those results. All of these results indicated that p85 α is a direct target of miR-29 and is negatively regulated by miR-29b in hepatocytes (Fig. 3). miR-29 inhibited PI3K pathway, and elevated the activation of gluconeogenesis.

We then compared the abundance of each miR-29 family members in the liver of C57BL/6 mice and primary hepatocytes. As shown in Fig. 4A, the expression of miR-29a was slightly higher than that of miR-29b or miR-29c. We found that the expression levels of all the three miR-29 family members significantly increased in the liver of mice during fasting (Fig. 4B). In contrast, both of mRNA and protein levels of p85 α decreased significantly in the liver of mice upon fasting. Since the expression levels of miR-29 family members and p85 α protein level were always conversely correlated in the liver of mice under different feeding conditions, these *in vivo* results further supported the notion that p85 α is a target of miR-29. Insulin signaling is repressed during fasting. Insulin receptor- β increased, and its downstream the phosphorylation of insulin-receptor, substrate IRS1, Akt reduced in skeletal muscle biopsies of volunteer subjects after fasting

(Wijngaarden et al. 2013). These results also indicated that the elevation of miR-29 expression and the repressed p85 α protein expression might facilitate the inhibition of insulin signaling in fasted status.

More importantly, miR-29 could repress the insulin-induced phosphorylation of Akt both in primary hepatocytes and mice Hepa1-6 hepatocytes, which suggested that miR-29b mimics was able to inhibit insulin signaling pathway. Further research showed that AICAR treatment enhanced the protein expression level of p85 α , which could be repressed by overexpression of miR-29b. These results further supported our hypothesis that miR-29 leads to the derepression of p85 α , which might mediate the effects of AICAR on insulin signaling (Fig. 6). This seems opposite to that of Liang et al. We speculated that it was because treatment of dexamethasone may mimic the pathologic station of insulin resistance. miR-29 may have different effects in physical and pathologic stations, which needs further investigation.

In this research, we conformed that AICAR treatment decreased the expression of all the miR-29 family members in C57BL/6 mouse primary hepatocytes, activated the PI3K-Akt pathway, hence insulin signaling pathway. AICAR treatment enhanced the protein level of p85 α , which could be repressed by overexpression miR-29b. These results further supported our hypothesis that miR-29 leads to the derepression of p85 α , which might mediate the effects of AICAR on insulin signaling. The *in vivo* results indicated that the elevation of miR-29 expression and the repressed p85 α protein level might facilitate the inhibition of insulin signaling in fasted status, which would be good for maintaining the

glucose homeostasis. Taken together, our results indicated that activation of AMPK might enhance insulin signaling via downregulation of miR-29.

Abbreviations AICAR, 5-aminoimidazole-4-carboxamide-1- β -d-ribofuranoside; Akt/PKB, serine-threonine protein kinase; AMPK, AMP-activated protein kinase; Bt₂-cAMP, dibutyryl-cAMP; IRS-1, insulin receptor substance 1; miRNAs, microRNAs; PI3K, phosphoinositide-3-kinase.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation (No. 81400784, 31371189) and Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (SIBS2012004).

Disclosure

None declared.

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Figure captions

Figure 1 Effects of AICAR on insulin signaling. (A) Expression levels of phosphorylation of AMPK in hepatocytes incubated with or without AICAR. (B) Expression levels of p-Akt in hepatocytes incubated with or without insulin or AICAR as indicated. (C) Protein expression levels of p85 α in hepatocytes incubated with or without insulin or AICAR as indicated.

Figure 2 Effects of AICAR on the expression of miR-29 family members in mouse primary hepatocytes. Relative expression of miR-29 family members was determined in C57BL/6 mouse hepatocytes incubated with different doses of AICAR (0.2mM or 0.4 mM for 6h). Results are representative of 3 independent experiments. Data were mean $\pm$ SD. * $P < 0.01$ compared with hepatocytes incubated without Bt₂-cAMP; # $P < 0.01$ compared with hepatocytes incubated with Bt₂-cAMP alone. Dex is short for dexamethasone.

Figure 3 miR-29b targets p85 α in C57BL/6 mouse primary hepatocytes. (A) Binding site of mmu-miR-29b on the 3'UTR of the p85 α regulatory subunit of PI3K. The seed region of miR-29b is complementary to residues 331~337 on the 3'UTR. Sequence alignment of the 3'UTR's of the p85 α subunit of PI3K across species. The conserved miR-29b seed region is shown within the white box. (B) The transfection of miR-29b mimics increased the expression of miR-29b ($n=3$). (C) Relative mRNA levels of p85 α expressed as fold activation after miR-29b mimics transfection ($n=3$). (D) Western blot analysis was performed by using p85 α antibody in primary hepatocytes transfected with

miR-29b mimics. (E) Relative mRNA levels of Pgc-1 α , Pepck and G6Pase expressed elevated after miR-29b mimics transfection ($n=3$). $*P < 0.05$, $**P < 0.01$ compared with control.

Figure 4 Expression of miR-29 family and p85 α under fasting and fed conditions in

C57BL/6 mouse liver. (A) There were no significant differences between the expression of miR-29 family members in the liver of C57BL/6 mice and primary hepatocytes ($n=3$). (B) The expression of miR-29 family members increased significantly in the liver of fasted C57BL/6 mice ($n=3-5$). (C) Relative mRNA levels of p85 α in the liver of fasted mice ($n=3$). (D) Protein levels of p85 α in the liver of fasted mice ($n=3$). Data was mean $\pm$ SD. $*P < 0.05$, $**P < 0.01$ compared with control.

Figure 5 Effects of miR-29 on insulin signaling. (A) Expression levels of p-Akt in C57BL/6 mouse primary hepatocytes incubated with or without insulin or miR-29b mimics. (B) Expression levels of p-Akt in Hepa 1-6 hepatocytes incubated with or without insulin or miR-29b mimics.

Figure 6 Effects of AICAR and miR-29 mimics on hepatocytes expression of p85 α .

Expression levels of p85 α in hepatocytes incubated with or without miR-29b mimics and AICAR.

Figure 1 Effects of AICAR on insulin signaling. (A) Expression levels of phosphorylation of AMPK in hepatocytes incubated with or without AICAR. (B) Expression levels of p-Akt in hepatocytes incubated with or without insulin or AICAR as indicated. (C) Protein expression levels of p85 α in hepatocytes incubated with or without insulin or AICAR as indicated.
99x119mm (300 x 300 DPI)

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175x184mm (300 x 300 DPI)

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70x59mm (300 x 300 DPI)

Figure 6 Effects of AICAR and miR-29 mimics on hepatocytes expression of p85 α . Expression levels of p85 α in hepatocytes incubated with or without miR-29b mimics and AICAR.
28x10mm (300 x 300 DPI)

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