FEBS Letters 584 (2010) 1503–1508

FEBS Letters

journal homepage: www.FEBSLetters.org

Ghrelin inhibits insulin secretion through the AMPK–UCP2 pathway in β cells

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ARTICLE INFO

Article history:
Received 29 December 2009
Revised 22 February 2010
Accepted 27 February 2010
Available online 3 March 2010

Edited by Robert Barouki

Keywords:
Ghrelin
AMP-activated protein kinase
Uncoupling protein 2
Insulin secretion

ABSTRACT

Ghrelin inhibits insulin secretion partly via induction of IA-2β. However, the orexigenic effect of ghrelin is mediated by the AMP-activated protein kinase (AMPK)–uncoupling protein 2 (UCP2) pathway. Here, we demonstrate that ghrelin's inhibitory effect on insulin secretion also occurs through the AMPK-UCP2 pathway. Ghrelin increased AMPK phosphorylation and UCP2 mRNA expression in MIN6 insulinoma cells. Overexpression or downregulation of UCP2 attenuated or enhanced insulin secretion, respectively. Furthermore, AMPK activator had a similar effect to ghrelin on UCP2 and insulin secretion in MIN6 cells. In conclusion, ghrelin's inhibitory effect on insulin secretion is partly mediated by the AMPK-UCP2 pathway, which is independent of the IA-2β pathway.

1. Introduction

Ghrelin, the only circulating orexigenic hormone, was first identified in rat stomach as an endogenous ligand of growth-hormone secretagogue receptor (GHSR) [1]. Although ghrelin is mainly produced and secreted from the stomach [1], pancreas also express ghrelin [2,3] and GHSR [4], as well as the pancreatic β cell line MIN6 cells [5]. In addition, ghrelin concentration is eight times higher in pancreatic veins than in pancreatic artery [6], suggesting that ghrelin is produced and released from islet cells and might act on β cells via autocrine and/or paracrine manner.

Besides modulating energy homeostasis by increasing food intake, body weight and adiposity [7,8], ghrelin was also suggested affecting pancreatic β cell function. Administration of ghrelin resulted in a decrease in plasma insulin and an increase in plasma glucose levels [9]. Overexpression of ghrelin led to inhibition of glucose-stimulated insulin secretion (GSIS) and deleting the gene of ghrelin or GHSR resulted in lower blood glucose [10] in mice. We have reported that ghrelin inhibited insulin secretion via inducing IA-2β [5]. But the intrinsic mechanism of this has not been investigated sufficiently.

AMP-activated protein kinase (AMPK) plays an important role in glucose homeostasis. It is well known that activation of AMPK suppresses GSIS in β cell lines [11] and isolated islets [12]. Ghrelin regulates AMPK activity in various tissues in a tissue-specific manner [13–15]. However, there is no report about how ghrelin affect AMPK activity in β cells till now.

Ghrelin also enhanced uncoupling protein 2 (UCP2) expression in the hypothalamus [13], liver [14] and white adipose tissue [8]. The orexigenic effect of ghrelin on the hypothalamus was demonstrated to be a UCP2-dependent action via AMPK [13]. Ghrelin-deficient mice showed reduced UCP2 mRNA expression and enhanced GSIS [16]. Overexpression of UCP2 in rat islets decreased insulin secretion [17]. Gonzalez-Barroso et al. reported an impaired activity of UCP2 mutants which was related with human congenital hyperinsulinism [18]. Therefore, ghrelin may affect insulin secretion through AMPK-UCP2 pathway.

2. Materials and methods

2.1. Cell culture and reagents

MIN6 cells were maintained in DMEM containing 25 mmol/l glucose, 10% FBS, and antibiotics (Invitrogen, Carlsbad, CA) at

Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; GHSR, growth-hormone secretagogue receptor; GSIS, glucose-stimulated insulin secretion; KRBH, Krebs–Ringer bicarbonate–Hepes buffer; PPAR, peroxisome proliferator-activated receptor; PGC-1α, PPAR-γ coactivator-1-alpha; QT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; siRNA, short-interfering RNA; UCP2, uncoupling protein 2

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0014-5793/$36.00
doi:10.1016/j.febslet.2010.02.069

37 °C in 5% CO_2. Ghrelin (acylated form) and desacyl-ghrelin were bought from Peptide Ins. (Osaka, Japan). 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was purchased from Sigma (St. Louis, MO). Antibodies for phospho-AMPK and total AMPK were obtained from Cell Signaling Technology (Danvers, MA).

2.2. Plasmid construction, RNA interference and transfection

Plasmids encoding mouse IA-2β were constructed as described elsewhere [5]; full length UCP2 clone obtained from the cDNA of MIN6 cells was subcloned into the plasmid pcDNA 3.1 (Invitrogen) at the EcoRI–NotI sites. Constructed plasmids were transfected to MIN6 cells using FuGENE6 Transfection Reagent (Roche Diagnostics, Mannheim, Germany). The pcDNA 3.1 vector was used as control. Short-interfering RNA (siRNA) of UCP2 was obtained from QIAGEN (Valencia, CA) and transfected into MIN6 cells by RNAiFect Transfection Reagent (QIAGEN). Negative-control siRNA (accompaniment to RNAiFect) was used as control.

2.3. RT-PCR and quantitative PCR

Total RNA derived from MIN6 cells was reverse-transcribed into cDNA and expression levels of UCP2 and IA-2β mRNA were analyzed by quantitative real-time PCR (QT-PCR) as described before [5]. Primer pairs and FAM-conjugated probes for UCP2 were purchased from Applied Biosystems (Assay ID: Mm00627599_ml). Data were calculated as copy number of each mRNA relative to ARP as an internal control.

2.4. Western blot analysis and AMPK activity

Cultured cells were washed twice with ice-cold PBS and resuspended immediately in lysis buffer containing 1% Non-diet P-40, 140 mmol/l NaCl, 20 mmol/l Tris–HCl (pH8.0), 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 1 mmol/l DTT, 10% glycerol, 0.5 mmol/l Na_3VO_4, 20 mmol/l pyrophosphate Na, 1 mmol/l PMSF, 5 mmol/l NaF, 1 mmol/l aprotinin, 4 mmol/l leupeptin, 5 mmol/l pepstatin. Protein content in the lysate was measured using the Bio-Rad Protein Assay kits (Bio-RAD, Hercules, CA). Protein samples (25 μg) were subjected to SDS–PAGE and transferred to nitrocellulose membrane then immunoblotted by using the phospho-AMPK and total AMPK antibodies. The proteins bound to antibodies were detected using horseradish peroxidase-conjugated anti-rabbit IgG (Biosource Int., Camarillo, CA) and visualized by using enhanced chemiluminescence detection system (MILLIPORE, Billerica, MA).

AMPK activity in the samples was assessed using the AMPK kinase assay kit (Cyclex, Nagano, Japan) according to the manufacturer's instructions.

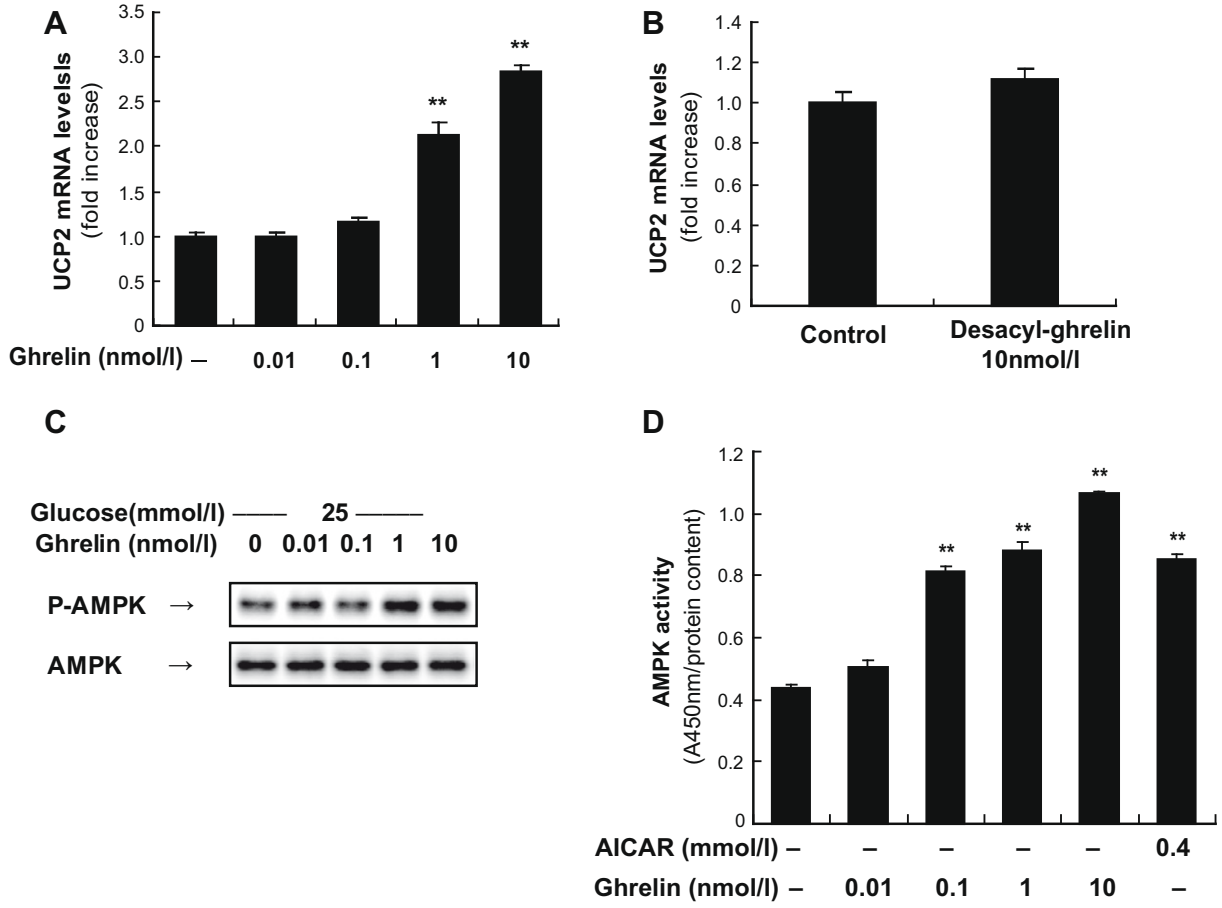

Fig. 1. Effect of ghrelin on UCP2 expression and AMPK activity in MIN6 cells. MIN6 cells were incubated 1 h under the following conditions: (A, C and D) with 0, 0.01, 0.1, 1, 10 nmol/l ghrelin; (B) with or without 10 nmol/l desacyl-ghrelin. (A and B) UCP2 mRNA expression levels were quantified by QT-PCR and expressed as fold increase relative to the values observed with cells that were not stimulated by ghrelin or desacyl-ghrelin. (C) Expression of phospho-AMPK (P-AMPK) and total AMPK (AMPK) were detected by Western blot analysis. The data presented is representative of three independent experiments. (D) AMPK activity was measured by AMPK kinase assay kit and expressed as absorbance at 450 nm and normalized to the protein content of the samples. All values are expressed as means ± S.E. of three independent experiments (n = 6–12, $^{*}P < 0.05$; $^{**}P < 0.01$ versus without ghrelin or desacyl-ghrelin).

2.5. Insulin secretion assay and insulin ELISA

The GSIS experiments were carried out as described before [5]. In short, MIN6 cells were incubated with indicated concentrations of glucose in the presence or absence of 10 nmol/l ghrelin or 0.4 mmol/l AICAR for one hour. Insulin content in the supernatant was quantified by an ELISA kit (Linco Research, St. Charles, MO) and normalized by the protein contents of the cell lysate. Data were expressed as ng/mg protein. To study the effect of UCP2 on insulin secretion, MIN6 cells were transfected with pcDNA3.1 UCP2 or UCP2 siRNA and the control vector or control siRNA 24hours before glucose and ghrelin stimulation.

2.6. Statistical analysis

Data are expressed as means ± S.E. for at least three independent experiments in duplicates. Variances in different groups were analyzed by Student's *t*-test or one-way ANOVA for unpaired comparisons. *P* value <0.05 was accepted as significant.

3. Results

3.1. Ghrelin upregulates UCP2 and activates AMPK in MIN6 cells

After the administration of ghrelin for 1 h at high glucose condition, UCP2 mRNA expression levels in MIN6 cells were upregulated dose-dependently (Fig. 1A). However, desacyl-ghrelin did not show any effect on UCP2 (Fig. 1B). Our data are consistent with the view that the acylation is essential for the bioactivity of ghrelin [1]. Ghrelin treatment also induced AMPK phosphorylation (Fig. 1C) as well as AMPK activity (Fig. 1D).

3.2. Effect of UCP2 overexpression or downregulation on insulin secretion

Overexpression of UCP2 attenuated GSIS in MIN6 cells with little or no effect on basal insulin secretion (Fig. 2A). Administration of ghrelin to the cells transfected with UCP2 for 1 h seemed to decrease GSIS further, but without statistical significance (Fig. 2B). On the other hand, downregulation of UCP2 by siRNA technique augmented GSIS in MIN6 cells and abolished ghrelin's inhibitory effect on GSIS (Fig. 2C).

3.3. AICAR elevates UCP2 expression and inhibits GSIS in MIN6 cells

Administration of AICAR (AMPK activator) for 1 h increased UCP2 mRNA expression levels dose-dependently (Fig. 3A), suggesting that AMPK might act upstream of UCP2 to mediate the effect of ghrelin on UCP2. AICAR suppressed the insulin secretion that was induced by 22.2 mmol/l glucose but left the basal insulin secretion intact (Fig. 3B), mimicking the effect of ghrelin. Administration of AICAR and ghrelin together to MIN6 cells decreased GSIS further in contrast to AICAR ($P < 0.05$) or ghrelin (not statistical significant) alone (Fig. 3C). Thus AMPK activation by ghrelin might play a role in the inhibitory effect of ghrelin on insulin secretion by lying between ghrelin and UCP2 and acting as a signal transmitter.

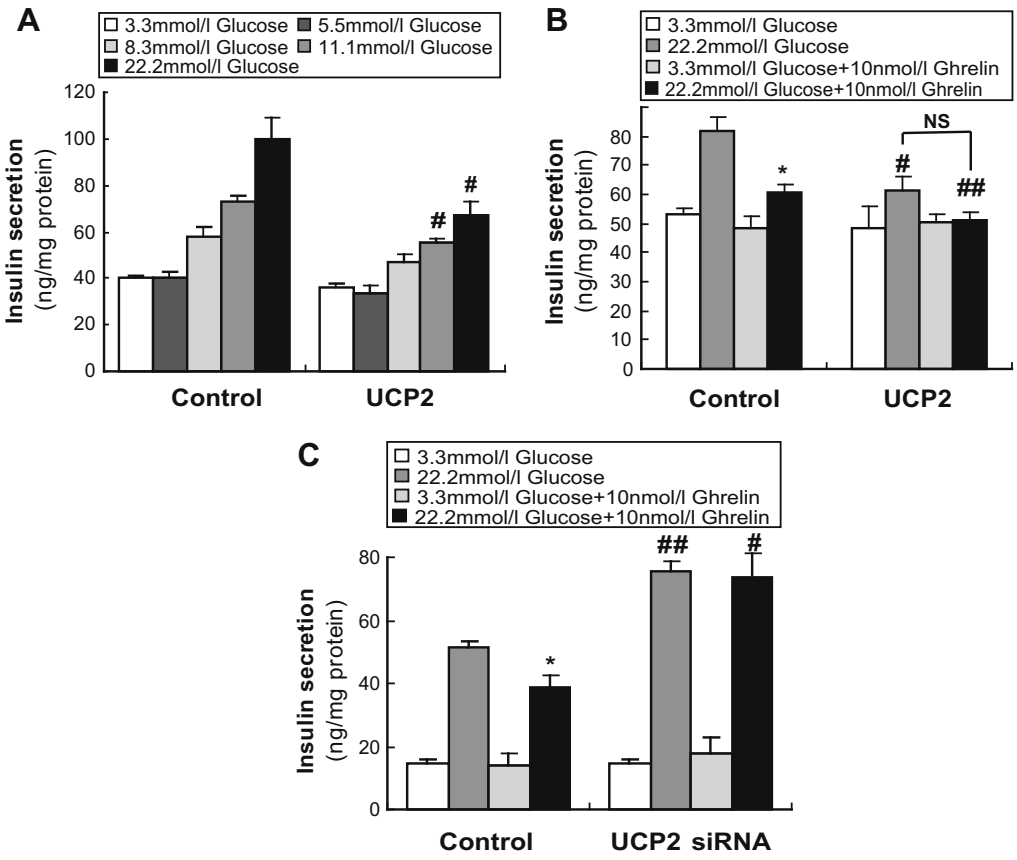

Fig. 2. Effect of UCP2 overexpression or downregulation on insulin secretion. (A and B) MIN6 cells transfected with constructed plasmid, pcDNA3.1 UCP2, or control vector were treated 1 h with (A) indicated concentrations of glucose (3.3, 5.5, 8.3, 11.1, 22.2 mmol/l); (B) 3.3 or 22.2 mmol/l glucose containing 10 nmol/l ghrelin or not. (C) MIN6 cells transfected with UCP2 siRNA or control siRNA were treated as described in (B). Insulin secreted into the medium was measured and normalized to the protein content of cell lysate. All values are expressed as means ± S.E. of three independent experiments ($n = 6–12$, $^{*}P < 0.05$ versus without ghrelin at the same condition. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ versus control vector or control siRNA transfection without ghrelin).

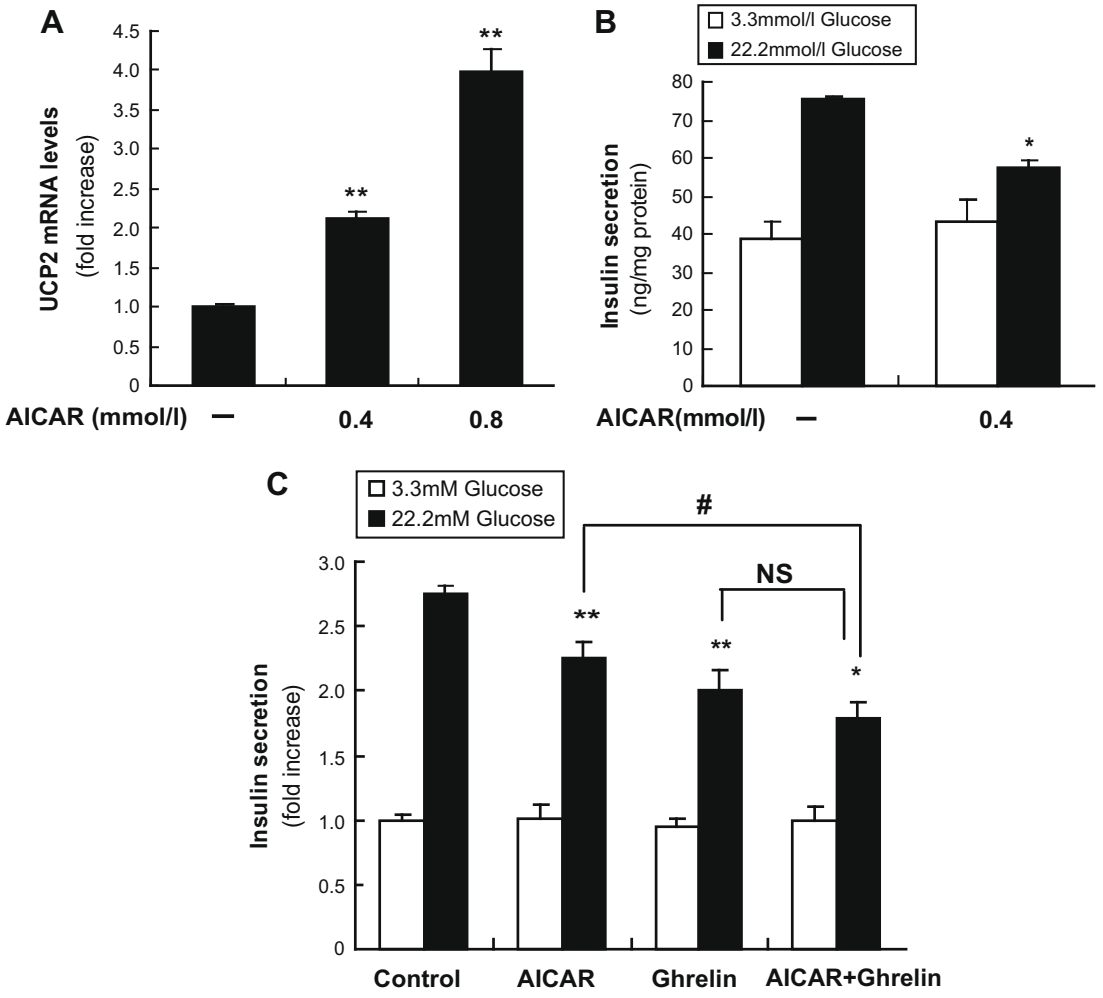

Fig. 3. Effect of AMPK activation by AICAR on UCP2 mRNA expression levels and insulin secretion. (A) UCP2 mRNA expression levels in MIN6 cells treated with AICAR (0, 0.4, 0.8 mmol/l) for 1 h. Data were expressed as fold increase relative to those observed without AICAR. (B) Insulin secretion in MIN6 cells treated 1 h with or without 0.4 mmol/l AICAR in the presence of 3.3 or 22.2 mmol/l glucose. Data were normalized to the protein content of cell lysate and expressed as ng/mg protein. (C) Insulin secretion in MIN6 cells treated with 0.4 mmol/l AICAR and 10 nmol/l ghrelin for 1 h. Data were expressed as fold increase relative to that obtained from the control cells incubated in 3.3 mmol/l glucose. All values are expressed as means ± S.E. of three independent experiments (n = 6–9, $^{*}P < 0.05$; $^{**}P < 0.01$ versus control not treated with AICAR and ghrelin. $^{\#}P < 0.05$ versus AICAR).

3.4. Interaction among AMPK, UCP2 and IA-2β

As we have reported that ghrelin inhibits GSIS via inducing IA-2β [5], we assumed that a crosstalk existed between the AMPK–UCP2 pathway and IA-2β pathway. But AMPK activation by AICAR failed to change IA-2β mRNA expression levels (Fig. 4A) in MIN6 cells. Moreover, overexpression of UCP2 did not affect IA-2β mRNA expression levels (Fig. 4B), and vice versa (Fig. 4C). These data suggest that there is not interaction between the two pathways.

4. Discussion

This study was designed to investigate the molecular mechanism of ghrelin's inhibitory effect on GSIS in pancreatic β cells. In this study, we found that ghrelin (acylated form) activates AMPK–UCP2 pathway in MIN6 cells. Furthermore, this pathway modulates GSIS. Therefore, this pathway plays a part in the inhibitory effect of ghrelin on insulin secretion.

Recently, UCP2 was suggested to regulate insulin secretion in many reports. We reported that the UCP2 promoter polymorphism −866G/A was related with GSIS and requirement of insulin therapy in Japanese type 2 diabetes [19]. Here by modulating UCP2 expression levels, we showed that UCP2 is closely related with insulin secretion and interfere with ghrelin's impact to MIN6 cells.

UCP2-deficient mice had higher islet ATP levels and increased GSIS [20]. On the contrary, overexpression of UCP2 in β cells abolished the inhibitory effect of glucose on KATP channel activity and diminished the glucose-stimulated increase of cytosolic Ca^{2+} concentration and insulin secretion [21].

AMPK activation increases the expression of UCP2 in liver [22], skeletal muscle [23], hypothalamus [13], and endothelial cells [24]. Here we found that activation of AMPK by AICAR upregulates UCP2 mRNA expression in MIN6 cells as well.

The mechanism through which AMPK increases UCP2 expression remains unclear. Peroxisome proliferator-activated receptor (PPAR) family, PPAR-α and PPAR-γ coactivator-1-alpha (PGC-1α) which have been described as regulators of mitochondrial biogenesis may be the possible mediators. It was suggested recently that the NAD^{+}-dependent type III deacetylase SIRT1 might lie between AMPK and PGC-1α [25]. On the other hand, reactive oxygen species (ROS) may be another choice. AMPK activation in β cells increased production of ROS, and increased ROS then promote UCP2 transcription and activity [13,26].

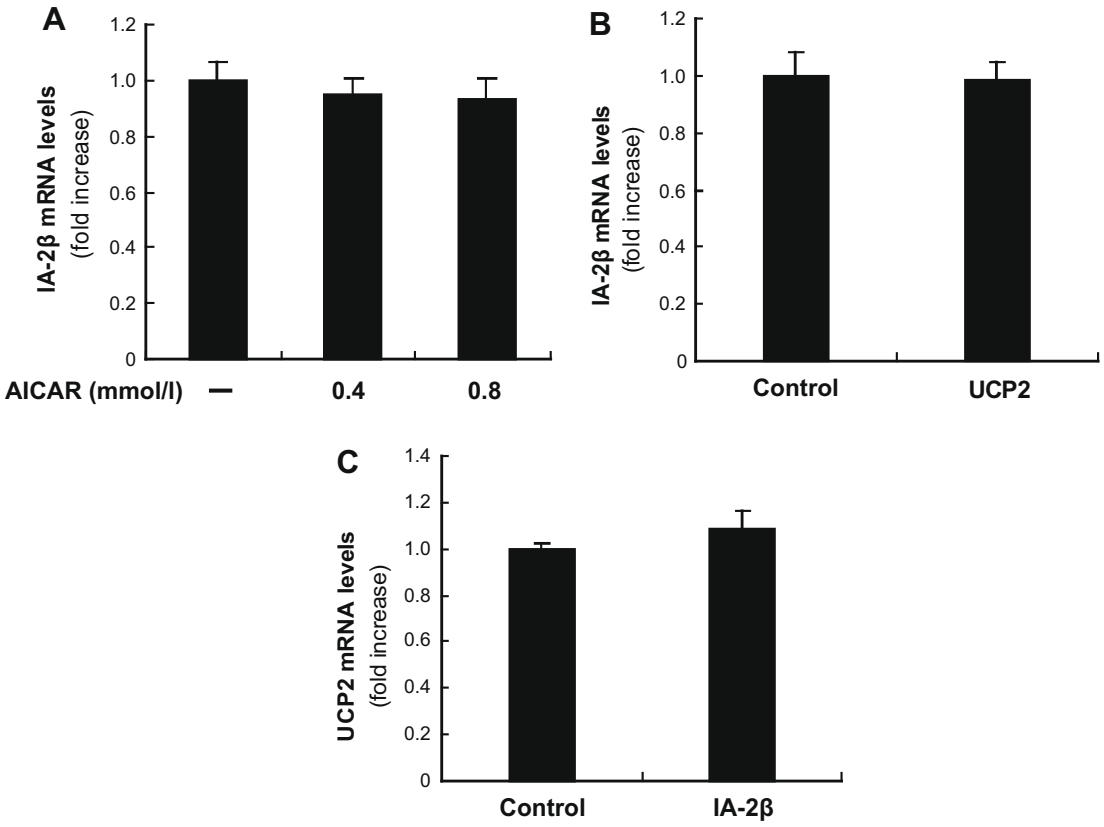

Fig. 4. Interaction among AICAR, IA-2β and UCP2. (A) MIN6 cells were treated with or without 0.4 mmol/l AICAR for 1 h, IA-2β mRNA expression levels were assessed and expressed as fold increase relative to those without AICAR. (B and C) MIN6 cells were transfected with pcDNA3.1 UCP2, pcDNA3.1 IA-2β or control vector respectively, (B) IA-2β and (C) UCP2 mRNA expression levels were measured 24 h after transfection. Data are expressed as fold increase relative to the values observed in cells transfected with control vector. All values are expressed as means ± S.E. of three independent experiments.

In keeping with the effect of ghrelin stimulation and UCP2 overexpression, AMPK activated by AICAR inhibited GSIS as well. As ghrelin activated AMPK induces UCP2 expression, it is possible that ghrelin upregulates UCP2 expression and inhibits GSIS via AMPK activation.

We noticed that AICAR and ghrelin together has a stronger effect on GSIS than AICAR or ghrelin alone, suggesting that they might have additive effect and might activate AMPK which was inhibited by high concentration of glucose [27] furthermore than one reagent alone. But this additive effect is only significant compared with AICAR alone, which could be explained by the existence of other pathways of ghrelin on insulin secretion such as IA-2β [5].

We previously reported that ghrelin inhibited insulin secretion via inducing IA-2β. However, neither did overexpression of UCP2 affect IA-2β mRNA expression nor did overexpression of IA-2β affect UCP2 mRNA expression. Likewise, AMPK activation by AICAR did not change IA-2β expression. Then it seems that IA-2β lies independent of the ghrelin–AMPK–UCP2 pathway. Therefore, ghrelin has intricate mechanisms that include different pathways in regulating insulin secretion.

5. Conclusions

Ghrelin activates AMPK and upregulates UCP2 mRNA expression in MIN6 cells. Furthermore, Ghrelin's inhibitory effect on insulin secretion is partly mediated by AMPK–UCP2 pathway which is independent of IA-2β pathway.

Acknowledgement

We thank Dr. Junichi Miyazaki (Osaka University) for kindly providing MIN6 cells.

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