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Activation of double-stranded RNA-dependent protein kinase inhibits proliferation of pancreatic β-cells

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ABSTRACT

Double-stranded RNA-dependent protein kinase (PKR) is revealed to participate in the development of insulin resistance in peripheral tissues in type 2 diabetes (T2DM). Meanwhile, PKR is also characterized as a critical regulator of cell proliferation. To date, no study has focused on the impact of PKR on the proliferation of pancreatic β-cells. Here, we adopted insulinoma cell lines and mice islet β-cells to investigate: (1) the effects of glucolipotoxicity and pro-inflammatory cytokines on PKR activation; (2) the effects of PKR on proliferation of pancreatic β-cells and its underlying mechanisms; (3) the actions of PKR on pro-proliferative effects of IGF-I and its underlying pathway. Our results provided the first evidence that PKR can be activated by glucolipitoxicity and pro-inflammatory cytokines in pancreatic β-cells, and activated PKR significantly inhibited cell proliferation by arresting cell cycle at G1 phase. Reductions in cyclin D1 and D2 as well as increases in p27 and p53 were associated with the anti-proliferative effects of PKR, and proteasome-dependent degradation took part in the reduction of cyclin D1 and D2. Besides, PKR activation abrogated the pro-proliferative effects of IGF-I by activating JNK and disrupting IRS1/PI3K/Akt signaling pathway. These findings indicate that the anti-proliferative actions of PKR on pancreatic β-cells may contribute to the pathogenesis of T2DM.

1. Introduction

Type 2 diabetes (T2DM) is currently viewed as a disease of relative insulin deficiency due not only to the dysfunction of pancreatic β-cells but also to the reduction of β-cell mass [1]. Pancreatic β-cell numbers are responsible for producing all of the insulin required by an organism to maintain glucose homeostasis, and deficiency in maintenance of β-cell mass results in the impairment of glucose metabolism and the occurrence of T2DM. Recent studies have demonstrated that maintenance of β-cell mass were mainly attributed to the proliferation of mature β-cells other than pluripotent stem cells in pancreas of mammals [2]. Hence, the proliferation of mature β-cells seems to play a crucial role in maintenance of glucose homeostasis under physiological and pathophysiological settings [3].

As a crucial molecule associated with lipid metabolism and inflammation, the double-stranded RNA-dependent protein kinase (PKR) is revealed to participate in the development of insulin resistance in peripheral tissues in T2DM [4]. Meanwhile, activation of PKR is also able to inhibit protein synthesis and cell proliferation during infection via multiple pathways [5]. However, whether activation of PKR contributed to the pathogenesis of T2DM by inhibiting the proliferation of pancreatic β-cells remained elusive. In this study, we adopted insulinoma cell lines and mice islet β-cells, to investigate: (1) the effects of glucolipotoxicity and pro-inflammatory cytokine on PKR activation; (2) the effects of PKR activation on proliferation of pancreatic β-cells and its underlying mechanism; (3) the actions of PKR activation on IGF-I-induced pro-proliferative effects and its underlying molecular pathway.

2. Materials and methods

2.1. Reagents, cell cultures and animals

The details of reagents, cell cultures and animals are depicted in Supplementary materials.

2.2. Islet purification and culturing

Islet isolation and culturing were performed as described previously [6]. The detailed protocol is presented in Supplementary materials.

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2.3. MTT and EdU incorporation assays

The detailed protocol of MTT and EdU incorporation assays is described in Supplementary materials.

2.4. Flow cytometry analysis of cell cycle and cell apoptosis

The detailed protocol of flow cytometry analysis is described in Supplementary materials.

2.5. Western blotting analysis and immunofluorescence assay

Western blotting analysis and immunofluorescence assay were conducted as previously described [7]. See Supplementary materials for detailed protocol.

2.6. Statistical analysis

The detailed protocol of *statistical analysis* is depicted in Supplementary materials.

3. Results

3.1. Glucolipitoxicity and pro-inflammatory cytokine lead to the activation of PKR

NIT-1 cells were subjected to the glucolipitoxicity stimulation (16.7 mM glucose and 0.4 mM palmitic acid) for 3, 6, 12 and 24 h. As shown in Fig. 1A, the p-PKR/PKR ratio significantly increased from 3 h, peaked at 6 h, began to decrease from 12 h, and was significantly lower than the baseline value at 24 h. Meanwhile, the ratio of p-eIF2α/eIF2α significantly increased from 6 h and reached its peak at 24 h. Then, we stimulated NIT-1 cells with pro-inflammatory cytokine (TNF-α, 80 nM) for 5, 10, 15 and 30 min. As demonstrated by Fig. 1C, the p-PKR/PKR ratio started to increase from 5 min, peaked at 10 min, began to drop from 15 min, and reached its bottom at 30 min. Meanwhile, the ratio of p-eIF2α/eIF2α reached its peak at 10 min, and lasted up to 15 min.

To investigate the role of PKR in the pancreatic β cells in response to glucolipitoxicity and pro-inflammatory cytokine, we pretreated NIT-1 cells with 2-AP (2.5, 5 and 10 mM) for 1 h, then stimulated them with glucolipitoxicity for another 6 h. As indicated by Fig. 1B, pretreatment with 2-AP abolished the glucolipitoxicity-induced PKR activation in a dose-dependent manner: for the p-PKR/PKR ratio, the reductions were significant at all doses (2.5, 5 and 10 mM). Meanwhile, the decreases in the ratio of p-eIF2α/eIF2α reached statistical significance at the doses of 5 and 10 mM. Afterwards, NIT-1 cells were subjected to the 2-AP pretreatment for 1 h and followed by stimulation with TNF-α (80 nM) for another 10 min. As expected, pretreatment with 2-AP reversed the pro-inflammatory cytokine-induced PKR activation in a dose-dependent fashion: for the ratios of p-PKR/PKR and p-eIF2α/eIF2α, the reductions reached statistical significance at the doses of 5 and 10 mM (Fig. 1D).

3.2. Activation of PKR inhibits proliferation through arresting cell cycle at G1 phase

We then stimulated INS-1 cells with BEPP for 48 h. As shown in Fig. 2A, BEPP caused a dose-dependent increase in p-eIF2α levels, and the changes were statistically significant at all three doses (2.5, 5 and 10 μM). This result was confirmed by immunofluorescence assay, which revealed that BEPP markedly increased p-eIF2α immunoreactivity in a dose-dependent manner (Fig. 2B).

Next, we stimulated INS-1 and NIT-1 cells with BEPP (2.5, 5, 10, 25, 50 and 100 μM) for 24, 48, and 72 h. As demonstrated by Fig. 2C, BEPP at the dose of 10, 25, 50 and 100 μM dose-dependently inhibited proliferation of INS-1 cells. Besides, BEPP (10, 25, 50 and 100 μM) fully abolished the time-dependent

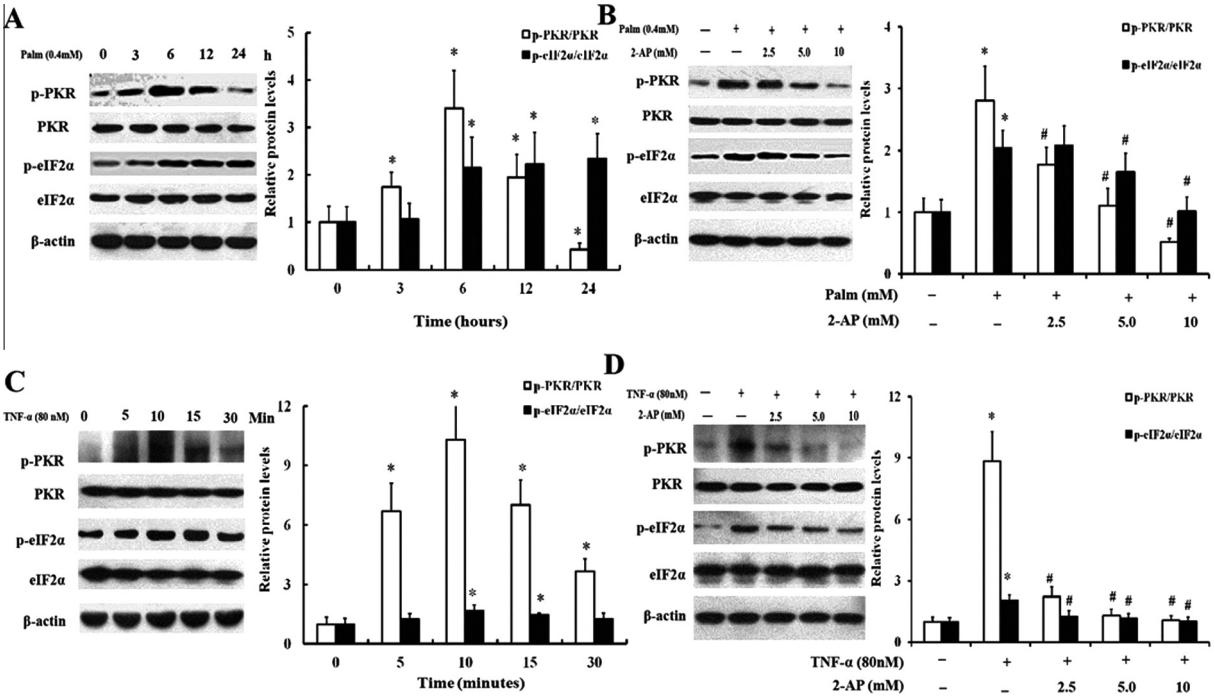

Fig. 1. Glucolipitoxicity and pro-inflammatory cytokine upregulate PKR activity in pancreatic β-cells. (A) Phosphorylation state of PKR and eIF2α after stimulation with glucolipitoxicity. $^{*}P < 0.05$ versus non-treated cells. (B) Phosphorylation state of PKR and eIF2α after pretreatment with 2-AP for 1 h and followed by glucolipitoxicity stimulation for 6 h. $^{*}P < 0.05$ versus non-treated cells, $^{\#}P < 0.05$ versus cells treated with palm only. (C) Phosphorylation state of PKR and eIF2α after stimulation with TNF-α. $^{*}P < 0.05$ versus non-treated cells. (D) Phosphorylation state of PKR and eIF2α after pretreatment with 2-AP for 1 h and followed by stimulation with TNF-α for 10 min. $^{*}P < 0.05$ versus non-treated cells, $^{\#}P < 0.05$ versus cells treated with TNF-α only. Data are presented as mean ± SEM.

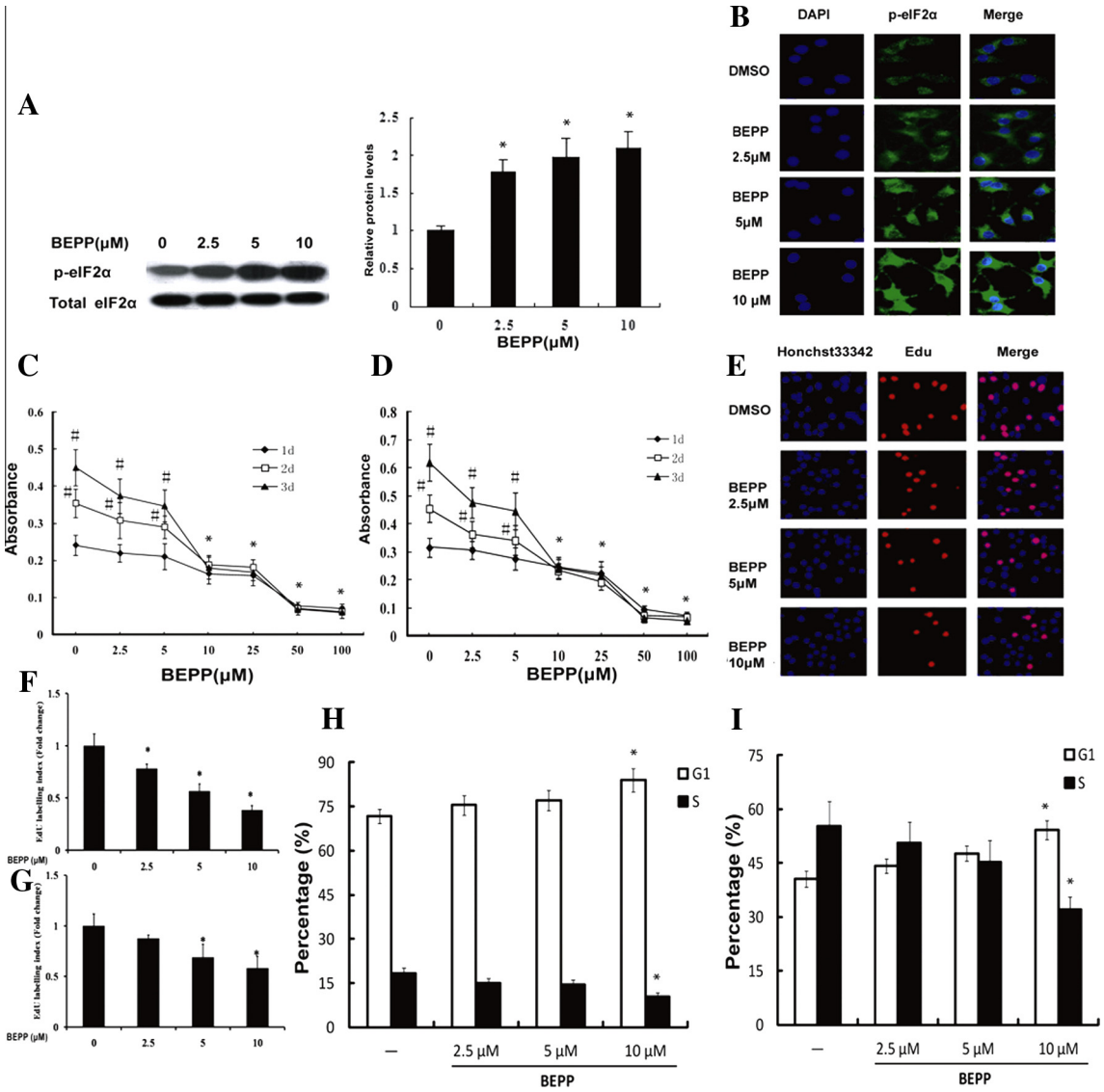

Fig. 2. Activation of PKR by BEPP inhibits proliferation through arresting cell cycle at G1 phase in pancreatic β-cells. (A) Phosphorylation state of eIF2α after treatment with BEPP for 48 h in INS-1 cells. $^{*}P < 0.05$ versus non-treated cells. (B) Phosphorylation state of eIF2α after treatment with BEPP for 48 h in INS-1 cells was confirmed by immunofluorescence assay α. (C, D) Proliferation of INS-1 and NIT-1 cells after treatment with BEPP was determined by MTT assay, $^{*}P < 0.05$ versus non-treated cells, $^{\#}P < 0.05$ versus cells received 24 h treatment. (E, F) Proliferation of INS-1 cells after treatment with BEPP for 48 h was determined by EdU incorporation assay. $^{*}P < 0.05$ versus non-treated cells. (G) Quantitative analysis of EdU-incorporated nuclei in islet β-cells after treatment with BEPP for 48 h. $^{*}P < 0.05$ versus non-treated cells. (H, I) The percentage of INS-1 and NIT-1 cells at G1 and S phase after stimulation with BEPP for 48 h was detected by flow cytometry. $^{*}P < 0.05$ versus non-treated cells. Data are presented as mean ± SEM.

proliferation of INS-1 cells, as there were no significant difference among absorbance at 24, 48, and 72 h. These results were replicated in NIT-1 cells, which showed that BEPP at the dose of 10, 25, 50 and 100 μM inhibited proliferation of NIT-1 cells in a dose-dependent manner. In addition, BEPP (10, 25, 50 and 100 μM) also inhibited the time-dependent proliferation of NIT-1 cells (Fig. 2D).

The results from MTT were confirmed by EdU incorporation assay. As demonstrated by Fig. 2E and F, treatment with BEPP for 48 h dose-dependently significantly reduced the number of EdU^{+} cells. This reduction reached statistical significance at all three doses (2.5, 5 and 10 μM). Meanwhile, treatment with 5 and 10 μM BEPP for 48 h significantly also inhibited the proliferation of islet β-cells by 31% and 42%, respectively (Fig. 2G).

Afterwards, cell cycle of INS-1 and NIT-1 cells was analyzed by flow cytometry. As revealed by Fig. 2H, treatment with BEPP for 48 h led to a dose dependent increase in percentage of INS-1 cells at G1 phase, and the difference reached significance at the doses of 10 μM. In addition, 10 μM BEPP caused a markedly reduction in percentages of INS-1 cells at S phase. Similarly, BEPP dose-dependently increased the percentages of NIT-1 cells at G1 phase, and the difference reached significance at the doses of 10 μM. Moreover, 10 μM BEPP led to a significant reduction in percentages of NIT-1 cells at S phase (Fig. 2I). Meanwhile, the inhibition of glucolipitoxicity- or pro-inflammatory cytokine-induced PKR activation on cell cycle was also confirmed by flow cytometry, as treated NIT-1 cells with 0.4 mM Palm or 80 nM TNF-α for 48 h remarkably arrested cell cycle at G1 phase (Fig. S1A and S1B).

3.3. Reductions of cyclin D1 and D2 levels as well as increases in p27 and p53 expression are associated with the PKR-induced arrest of cell cycle

We then examined the alteration of some cell cycle regulatory protein in response to PKR activation. As demonstrated by Fig. 3A, treated INS-1 cells with BEPP (2.5, 5 and 10 μM) for 48 h

significantly reduced the levels of cyclin D1 in a dose-dependent fashion. Meanwhile, BEPP at the dose of 5 and 10 μM caused a significant decrease in cyclin D2 levels. However, BEPP at current doses (2.5, 5 and 10 μM) did not markedly affect the expression of cyclin A2, cyclin E, and cyclin D3. In addition, BEPP increased p27 and p53 levels in a dose-dependent manner, and the changes were statistically significant at the doses of 5 and 10 μM. Moreover, activation of PKR by glucolipitoxicity or pro-inflammatory cytokine caused the similar effects on these cell cycle regulatory proteins in comparison to BEPP, as cyclin D1 and cyclin D2 were dramatically reduced while p53 and p27 were significantly increased in NIT-1 cells after treatment with 0.4 mM Palm or 80 nM TNF-α for 48 h (Fig. S1C and S1D).

To clarify the mechanisms underlying PKR-induced cell cycle arrest, INS-1 cells were subjected to proteasome inhibitor MG132 (2.5, 5 and 10 μM) for 1 h, and then received BEPP simulation (10 μM) for 48 h. As shown in Fig. 3B, MG132 dose-dependently rescued the BEPP-induced reductions in cyclin D1 and D2 levels, and the differences reached statistical significance at the doses of 5 and 10 μM. In addition, to investigate whether PKR caused cell cycle arrest via damaging cell DNA, several DNA damage checkpoints including ratios of p-CHK1/CHK1, p-CHK2/CHK2, p-P53/P53, and p-CDC2/CDC2 as well as protein levels of P21 and γ-H2AX were assessed. As indicated by Fig. S2, BEPP treatment did not significantly affect aforementioned DNA damage checkpoints.

3.4. Activation of PKR attenuates IGF-I-induced proliferation and cell cycle progression

INS-1 cells were subject for starvation for 24 h in medium depleted in amino acids and serum, followed by IGF-I treatment in serum-free medium containing 16.7 mM glucose. As indicated by Fig. 4A, IGF-I led to a reduction in percentage of cells at G1 phase as well as an increase in percentage of cells at S phase. Meanwhile, the differences were statistically significant at the doses of 10 and 50 nM. To explore role of PKR in IGF-I-induced cell proliferation, we co-treated INS-1 cells with IGF-I and BEPP. As demonstrated by Fig. 4B, co-treatment with BEPP (10 μM) fully abolished the progression of cell cycle induced by IGF-I (10 nM). In addition, MTT assay revealed that IGF-I (10 nM) significantly stimulated cell proliferation, which was markedly inhibited by BEPP in a dose-dependent manner at the doses of 10, 25 and 50 μM (Fig. 4D). The results from MTT assay were confirmed by EdU incorporation assay, as co-treatment INS-1 cells with IGF-I (10 nM) and BEPP (2.5, 5 and 10 μM) for 24 h significantly reduced

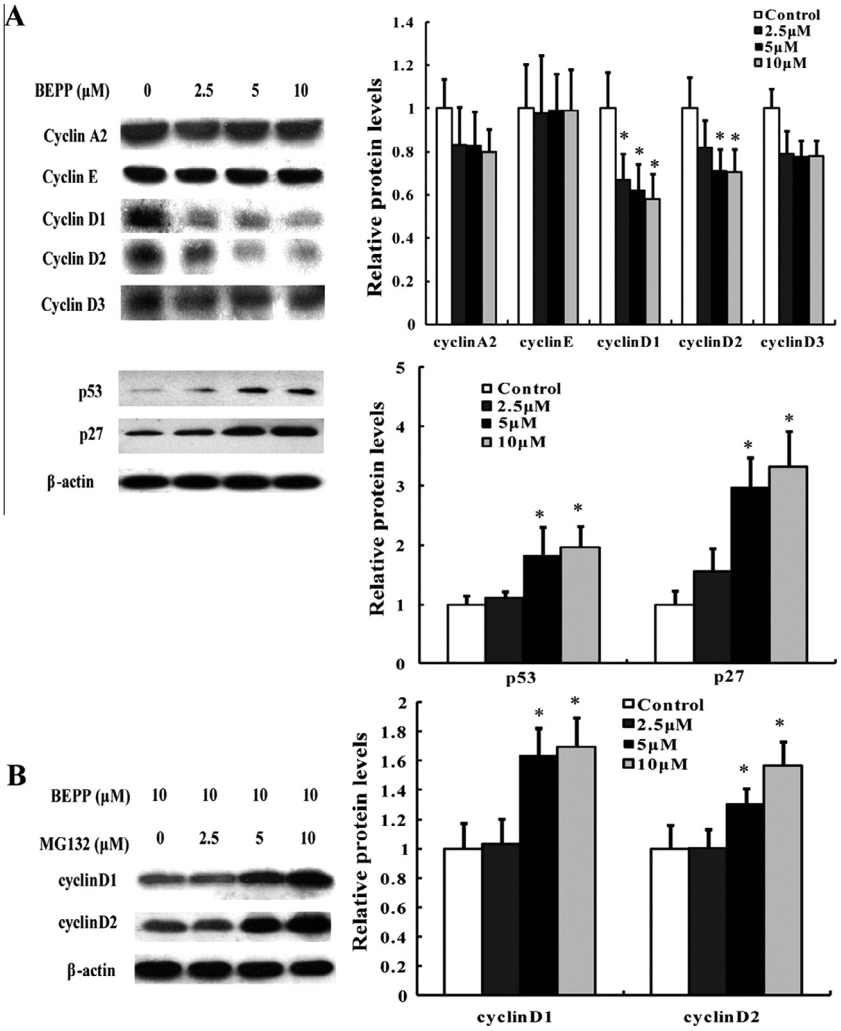

Fig. 3. Reductions of cyclin D1 and D2 levels as well as increases in p27 and p53 expression are associated with the BEPP-induced arrest of cell cycle in INS-1 cell. (A) Protein levels of cyclin A2, cyclin E, cyclin D1, cyclin D2, cyclin D3, p27, and p53 after treatment with BEPP for 48 h were evaluated by western blotting. (B) Protein levels of cyclin D1 and D2 in INS-1 cells after pretreatment with MG132 and followed by BEPP simulation for 48 h were detected by western blotting. *$P < 0.05$ versus non-treated cells. Data are presented as mean ± SEM.

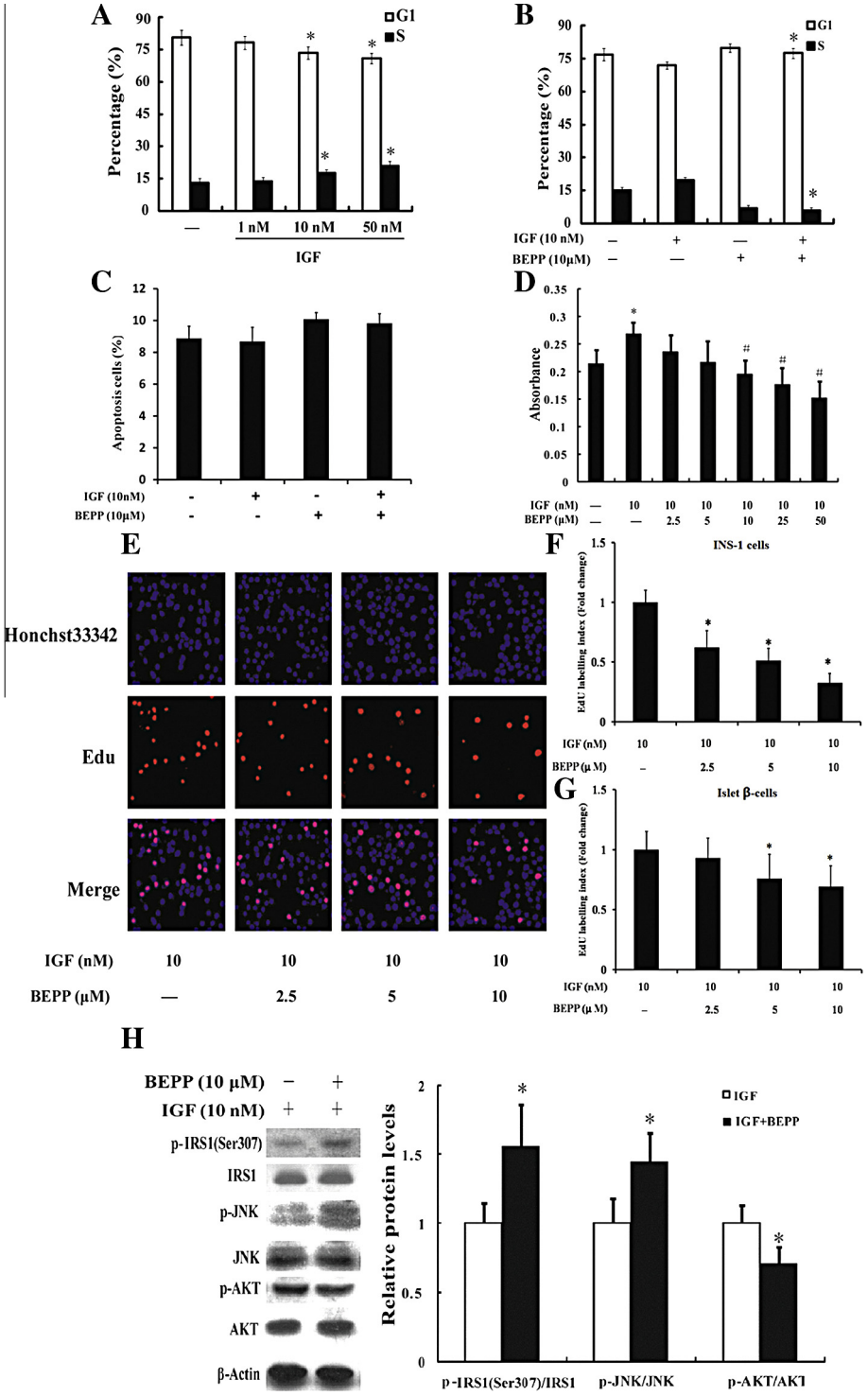

Fig. 4. Activation of PKR by BEPP attenuates IGF-I-induced proliferation and cell cycle progression in INS-1 cell. (A) The percentage of INS-1 cells at G1 and S phase after stimulation with IGF-I for 24 h was detected by flow cytometry. $^{*}P < 0.05$ versus non-treated cells. (B) The percentage of INS-1 cells at G1 and S phase after co-treatment with IGF-I and BEPP for 24 h was detected by flow cytometry. $^{*}P < 0.05$ versus cells treated with IGF-I only. (C) The apoptosis rate in INS-1 cells after co-treatment with IGF-I and BEPP for 24 h was detected by flow cytometry. (D) Proliferation of INS-1 cells after co-treatment with IGF-I and BEPP for 24 h were determined by MTT assay. $^{*}P < 0.05$ versus non-treated cells; $^{\#}P < 0.05$ versus cells treated with IGF-I only. (E, F) Proliferation of INS-1 cells after co-treatment with IGF-I and BEPP for 24 h was determined by EdU incorporation assay. $^{*}P < 0.05$ versus cells treated with IGF-I only. (G) Quantitative analysis of EdU-incorporated nuclei in islet β-cells after co-treatment with IGF-I and BEPP for 24 h. $^{*}P < 0.05$ versus cells treated with IGF-I only. (H) Phosphorylation and total levels of IRS1, JNK, and Akt in INS-1 cells after co-treatment with IGF-I and BEPP for 24 h was measured by western blotting. $^{*}P < 0.05$ versus cells treated with IGF-I only. Data are presented as mean ± SEM.

the number of EdU^{+} cells in a dose-dependent pattern (Fig. 4E and F). Meanwhile, co-treated pancreatic islets with IGF-I (10 nM) and BEPP (2.5, 5 and 10 μM) for 24 h dose-dependently attenuated the IGF-I-induced proliferation of islet β-cells, and the reduction reached statistical significance at the doses of 5 and 10 μM (Fig. 4G). It should be noted that treated INS-1 cells with IGF-I (10 nM) or BEPP (10 μM) alone did not affect the apoptosis rate of INS-1 cells. In addition, although co-treated INS-1 cells with

IGF-I (10 nM) and BEPP (10 μM) tended to increase the apoptosis rate in comparison to cells treated with IGF-I (10 nM) alone, the difference was not statistically significant (Fig. 4C).

To study the signaling pathways involved in the inhibition of IGF-I-induced proliferation, the ratios of p-IRS1(Ser307)/IRS1, p-JNK/JNK as well as p-Akt/Akt were evaluated. As shown in Fig. 4H, co-treatment INS-1 cells with IGF-I (10 nM) and BEPP (10 μM) for 24 h markedly increased the ratios of p-IRS1 (Ser307)/IRS1 and p-JNK/JNK when compared with cells treated with IGF-I alone (10 nM). Besides, it also led to a significant reduction in p-Akt/Akt ratio.

4. Discussion

In the current study, we provided the first evidence that stimulation with glucolipitoxicity and pro-inflammatory cytokine led to the activation of PKR pathway in pancreatic β-cells. These results are consistent with a previous study by Carvalho-Filho et al., which revealed that PKR is activated in peripheral tissues of obese mice with hyperglycemia [4]. Meanwhile, von Holzen and colleagues found that overexpression of TNF-alpha led to PKR upregulation in human esophageal cancer cell lines, providing further support to our results [8]. Afterward, we observed that PKR activation significantly inhibited the proliferation of pancreatic β-cells, and this anti-proliferative effect seemed to be accomplished by blocking cell cycle at G1 phase. Pancreatic β-cells are responsible for producing all of the insulin required by an organism to maintain glucose homeostasis. Besides, pancreatic β-cells have capacity to compensate for insulin resistance in peripheral tissues by increasing insulin production and secretion. However, reduction of β-cell numbers caused by inhibition of proliferation will lead to the loss of compensatory ability, and subsequently contribute to the glucose intolerance. Therefore, PKR-induced inhibition of β-cell proliferation seems to be a crucial factor in the pathogenesis of T2DM.

To investigate the mechanism by which activation of PKR arrested cell cycle at G1 phase, we measured the expression of cell cycle regulating factors. As expected, cyclin D1 and D2, the positive regulators of cell cycle, was decreased after PKR activation. Meanwhile, increases in p27 and p53, the negative controllers of cell cycle, were also observed. Cyclin D1 is able to stimulate proliferation by driving cells from the G1 into the S-phase of the mammalian cell cycle. Overexpression of cyclin D1 can induce proliferation of β-cells in vivo [9], whereas *cyclin D1*$^{-/-}$ mice have a severe islet growth deficiency that causes death from uncontrolled diabetes [10]. Similarly, cyclin D2 is also essential for normal islet growth and glucose homeostasis throughout life. This requirement was proven by *cyclin D2*$^{-/-}$ mice, which progressed to diabetes due to the deficiency in β-cell proliferation [10]. P27 is considered as G1-checkpoint CDK inhibitors, which prevents cell-cycle progression into the S phase. Repression of p27 contributed to the cell growth in Lung [11]. Conversely, overexpression of p27 significantly inhibited proliferation of MCF-7 cells, followed by accumulation of MCF-7 cells in cycle G1 [12]. P53 is a well-known tumor suppressor gene in mammal cells [13]. Expression of full-length p53 induces G1 arrest, which leads to an irreversible inhibition of cell growth, most decisively by activating apoptosis [14]. These results indicated that both down-regulation of cyclin D1 and D2 and up-regulation of p27 and p53 are related to the PKR-induced arrest of cell cycle at G1 phase. Considering the fact that 26S proteasome pathway has been found to destruct cell cycle regulators and promote the progression of cell cycle [15], we then adopted proteasome inhibitor MG132 to elucidate the underlying pathway by which reduced the levels of cyclin D1 and D2. Not surprisingly, the PKR-induced reduction in cyclin D1 and D2 were rescued by MG132, suggesting the decrease of cyclin D1 and D2 was mainly attributed to the enhancement of degradation. These results were in consistent with a recent study from Raven et al., which revealed that PKR-induced reduction in cell cycle regulators was mainly due to the promoted degradation, rather than translation inhibition [16]. It should be noted that PKR activation may arrest cell cycle at G1 phase via other mechanisms, such as damaging cell DNA [17]. However, in the current study, activation of PKR did not significantly affect DNA damage checkpoint proteins, excluding the possibility that PKR blocked cell cycle of pancreatic β-cells via induction of DNA damage.

Insulin-like growth factor I (IGF-I) is a growth factor structurally related to insulin, interacts with insulin-like growth factor receptor 1 (IGF-IR), and provides beneficial effects on glucose homeostasis [18]. Mounting evidence suggested a pro-proliferative effect of IGF-I, as IGF-I was found to promote the proliferation of cancer cells through activation of IRS1/PI3K/Akt signaling pathway [19–21]. In this study, we extended their findings by showing that IGF-I stimulated proliferation of INS-1 cells through driving them from the G1 into the S-phase.

Interestingly, we found that the pro-proliferative effects of IGF-I on pancreatic β-cells were fully abrogated by PKR activation. Concomitantly, an increase in phosphorylated IRS1 at Ser 307 and a reduction in phosphorylated Akt were observed, suggesting the IRS1/PI3K/Akt signaling pathway was disrupted. As one of the important down-stream effectors of the IGF-IR signaling pathway, IRS1 is crucial for the IGF-IR-induced activation of the PI3K/Akt pathways [22]. The phosphorylation of IRS1 plays critical roles in determining its activity, and Ser 307 has been extensively investigated and characterized as a key inhibitory phosphorylation site of IRS1 in murine models [23]. Based the above evidence, the anti-proliferative actions of PKR in the current study may be mediated by disrupting IRS1/PI3K/Akt signaling pathway.

Meanwhile, a moderate but significant increase in phosphorylated JNK, a translation factor regulated by PKR, was noted in this study. As one of the major down-stream targets of insulin signaling, JNK has been shown to function as IRS1 serine kinase, which are involved in the negative feedback pathways of insulin signaling by promoting the inhibitory phosphorylation of IRS1 at Ser 307 in rodents and at Ser 312 in humans (equivalent to Ser 307 in rodent IRS1) [24,25]. Meanwhile, Yang et al. found that activated PKR upregulated the inhibitory phosphorylation of IRS1 at Ser 312 in human hepatoblastoma cells by activating JNK [26]. Hence, the PKR-induced disruption of IRS1/PI3K/Akt signaling pathway was likely due to the activation of JNK in this study.

In summary, this study demonstrates that glucolipitoxicity and pro-inflammatory cytokine can activate PKR in pancreatic β-cells, leading to the inhibition of proliferation through arresting cell cycle at G1 phase. Reductions in cyclin D1 and cyclin D2 and increases in p27 and p53 proteins may be responsible for the anti-proliferative effects of PKR. Meanwhile, proteasome-dependent degradation is found to take part in the reduction of cyclin D1 and cyclin D2 proteins. Besides, PKR also abrogates the pro-proliferative effects of IGF-I through activating JNK and subsequently disrupting IRS1/PI3K/Akt pathway. These findings indicate that the anti-proliferative action of PKR on pancreatic β-cells may contribute to the pathogenesis of T2DM.

Conflict of interest

None declared.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.051.

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