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AMPK regulates K_{ATP} channel trafficking via PTEN inhibition in leptin-treated pancreatic β-cells

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ABSTRACT

Leptin regulates pancreatic β-cell excitability through AMP-activated protein kinase (AMPK)-mediated ATP-sensitive potassium (K_{ATP}) channel trafficking. However, the signaling components connecting AMPK to K_{ATP} channel trafficking are not identified. In this study, we discovered that AMPK inhibits phosphatase and tensin homologue (PTEN) via glycogen synthase kinase 3β (GSK3β) and this signaling pathway is crucial for K_{ATP} channel trafficking in leptin-treated pancreatic β-cells. Pharmacologic or genetic inhibition of AMPK or GSK3β, but not casein kinase 2 (CK2), impaired leptin-induced PTEN inactivation and thereby K_{ATP} channel trafficking. The PTEN mutant lacking both protein and lipid phosphatase activity is sufficient to induce K_{ATP} channel trafficking without leptin. These results present a novel signaling mechanism that underlies leptin regulation of K_{ATP} channel trafficking in pancreatic β-cells. Our findings assist in gaining a broader perspective on the peripheral action of leptin on pancreatic β-cell physiology and glucose homeostasis.

1. Introduction

Leptin is an adipocyte-derived hormone that controls food intake, glucose homeostasis, and energy expenditure in response to the amount of body fat [1,2]. In addition to its central action, leptin directly affects pancreatic β-cell functions at different levels involving insulin secretion and cell viability [3–7]. The defect in leptin signaling in pancreatic β-cells leads to uncontrolled insulin secretion and a failure of glucose homeostasis [1,8,9]. Therefore, unraveling the leptin signaling pathways in pancreatic β-cells may hold key to explaining the epidemiological link between obesity and diabetes.

ATP-sensitive potassium (K_{ATP}) channel, which comprises pore-forming Kir6.2 and regulatory SUR1 subunits, plays a crucial role in regulating pancreatic β-cell excitability and insulin secretion [10]. Recently, trafficking of K_{ATP} channels to the plasma membrane has emerged as an important mechanism controlling pancreatic β-cell excitability [11]. We also found that leptin causes K_{ATP} channel trafficking in pancreatic β-cells via calmodulin-dependent protein kinase kinase β (CaMKKβ)-dependent activation of AMP-activated protein kinase (AMPK) [7,12], providing a molecular mechanism for previous observations that leptin activates K_{ATP} channels [5,7,13]. Therefore, a mechanistic understanding of leptin-induced K_{ATP} channel trafficking will provide a new perspective on the development of obesity-associated diabetes.

Phosphatase and tensin homologue (PTEN) controls not only cell growth and survival, but also metabolic signaling [14]. Comparable to leptin, several lines of evidence indicate that PTEN is involved in the regulation of insulin secretion and glucose homeostasis: suppression of PTEN expression reduces serum insulin levels in *ob*/*ob* mice [15]; and PTEN loss enhances pancreatic β-cell growth and survival [16,17]. In addition, a previous report argued a functional relationship between leptin and PTEN in pancreatic β-cells: leptin leads to PTEN inhibition and K_{ATP} channel activation [18]. These results raise the possibility that leptin-induced PTEN inhibition is mediated by AMPK, but the link between PTEN and AMPK has never been identified. In this study, we discovered that leptin inhibits PTEN through AMPK-mediated activation of glycogen synthase kinase 3β (GSK3β) and this pathway is crucial for K_{ATP} channel trafficking. Our results will provide insight into the molecular mechanisms that underlie leptin action on pancreatic β-cell physiology and glucose homeostasis.

Abbreviations: PTEN, phosphatase and tensin homologue; AMPK, AMP-activated protein kinase; GSK3β, glycogen synthase kinase 3β; K_{ATP} channel, ATP-sensitive potassium channel; CK2, casein kinase 2; mTORC1, mammalian target of rapamycin complex 1; TSC2, tuberous sclerosis 2.

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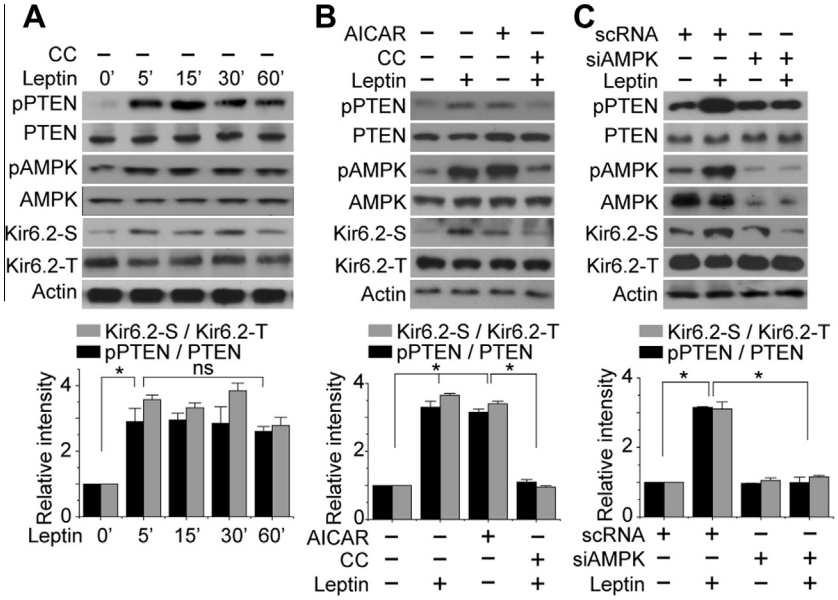

Fig. 1. AMPK mediates leptin-induced PTEN inactivation in INS-1 cells. (A and B) Cells were treated with 10 nM leptin and/or 10 μM CC or 250 μM AICAR for the indicated time or 30 min prior to Western blot analysis. Cells were treated with 10 nM leptin for 30 min prior to surface biotinylation. Surface (*S*) and total (*T*) fractions were probed using anti-Kir6.2 antibody. (C) Cells were transfected with scrambled siRNA (scRNA) or siAMPK for 48 h and then treated with 10 nM leptin for 30 min. The relative ratios of pPTEN to total PTEN or surface to total Kir6.2 were plotted based on the quantification of the band intensities. The data are expressed as the mean ± SEM ($n = 3$). $^{*}p < 0.05$.

2. Materials and methods

2.1. Cell culture and reagents

Insulin-secreting INS-1 cells (passage 20–50) and primary pancreatic β-cells were grown as described in our previous paper [19]. All animal experimental procedures for isolating pancreatic β-cells were conducted in accordance with the guidelines of the University Committee on Animal Resources at Seoul National University (Approval No.: SNU-120216-02). All cell culture reagents were purchased from Invitrogen. The cells were treated with leptin (Calbiochem), AICAR (Calbiochem), compound C (CC) (Calbiochem), CT99021 (Calbiochem), Kenpaullone (Calbiochem), and/or 4,5,6,7-tetrabromobenzotriazole (TBB) (Tocris). All other reagents not specified were supplied by Sigma–Aldrich.

2.2. Transfection experiment

Transfection experiments using siRNAs were performed as described in our previous paper [19]. PTEN constructs were provided by Professor Jae Ho Kim (Pusan National University). INS-1 cells were transfected with PTEN constructs for 48 h prior to the indicated analyses.

2.3. Surface level quantification

The surface levels of Kir6.2, a subunit of K_{ATP} channel, were analyzed using EZ-Link Sulfo-NHS-SS-Biotin kit (Pierce) as described in our previous paper [19].

2.4. Western blot analysis

The protein samples were resolved by 6–12% SDS–PAGE. Antibodies to Kir6.2, GSK3β, pGSK3β^{Ser9}, and PTEN were purchased from Santa Cruz Biotechnology. Antibodies to actin, CK2, pCK2^{Tyr255}, GAPDH, pGSK3^{Tyr216}, and pPTEN$^{Ser380/Thr382/Thr383}$ were obtained from Abcam. Antibodies to AMPK, pAMPK^{Thr172}, ACC, pACC^{Ser79}, AKT, and pAKT^{Ser473} were supplied by Cell Signaling.

2.5. Patch clamp analysis

Patch clamp experiments were performed as described in our previous paper [19]. Briefly, K_{ATP} channel currents was measured at room temperature using standard with an EPC-8 amplifier and followed by analysis with IGOR software. Patch electrodes were pulled from borosilicate glass capillaries to make the resistance ranged between 3 and 5 MΩ when filled with pipette solution. The internal solution for whole-cell experiments contained the following (in mM): 30 KCl, 110 K-aspartate, 2.6 $CaCl_2$, 10 HEPES (pH 7.2 with KOH), 0.5 EGTA and 5 EDTA. Free Ca^{2+} concentration was calculated to be about 24 nM. EDTA was added to minimize K_{ATP} current rundown [20]. The bath solution was composed of the following (in mM): 137 NaCl, 5.6 KCl, 10 HEPES (pH 7.4 with NaOH), 0.5 $MgCl_2$, and 1.8 $CaCl_2$. The cells were treated with the indicated reagents during or before the current recordings.

2.6. Immunofluorescence analysis

The cells were fixed with 4% paraformaldehyde in PBS for 15 min and then permeabilized with 0.25% Triton X-100 in PBS for 10 min at room temperature. After blocking with 2% donkey serum in PBS at room temperature for 30 min, the cells were incubated with anti-Kir6.2 antibody for 16 h at 4 °C. The subcellular localization of Kir6.2 was assessed using Alexa488-conjugated anti-rabbit IgG antibody (Invitrogen). The cells were photographed with a FluoView 1000 confocal microscope (Olympus).

2.7. Statistical analysis

The data were expressed as the mean ± SEM. Comparison of mean values among experimental groups was performed using one-way ANOVA followed by a post hoc test. $p < 0.05$ was considered statistically significant.

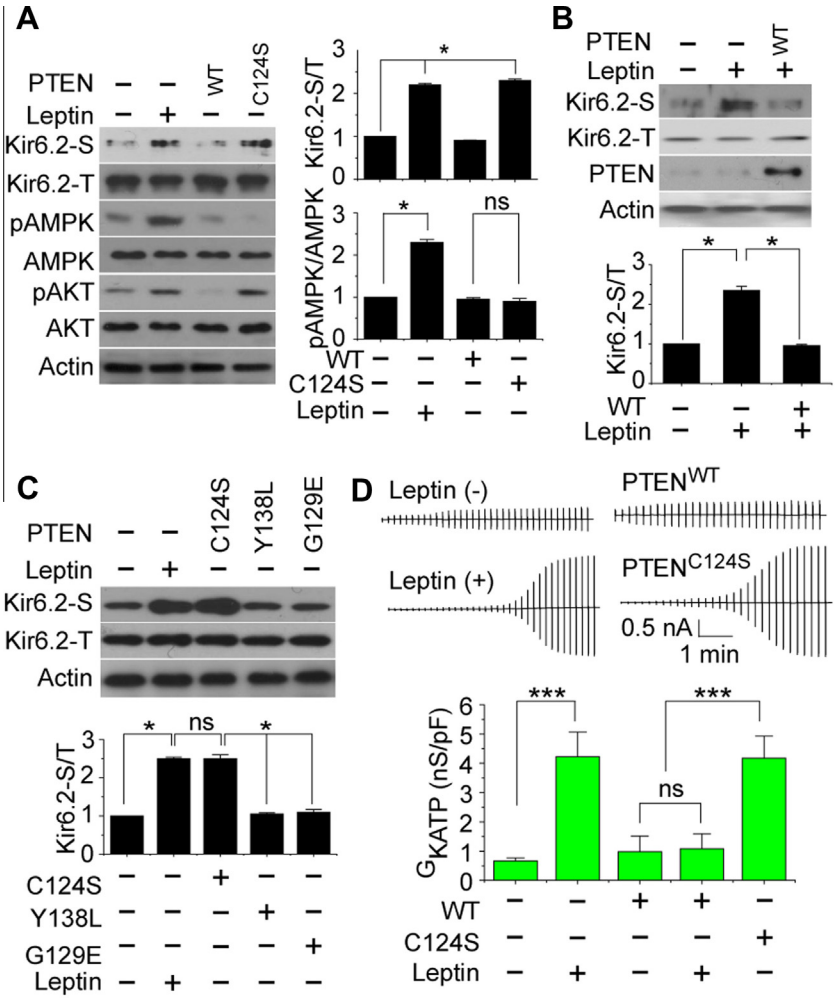

Fig. 2. AMPK-mediated PTEN inhibition increases K_{ATP} channel surface expression in INS-1 cells. (A) Cells were transfected with $PTEN^{WT}$ or $PTEN^{C124}$ construct for 48 h and then treated with 10 nM leptin for 30 min prior to surface biotinylation. The relative ratios of surface to total Kir6.2 and pAMPK to total AMPK were plotted based on the quantification of the band intensities. The data are expressed as the mean ± SEM ($n = 3$). $^{*}p < 0.05$; ns, not significant. (B) Cells were transfected with $PTEN^{WT}$ for 48 h and then treated with leptin for 30 min prior to surface biotinylation. The data are expressed as the mean ± SEM ($n = 3$). $^{*}p < 0.05$. (C) Cells were transfected with $PTEN^{WT}$ or mutant PTEN constructs and then treated with 10 nM leptin for 30 min prior to surface biotinylation. The data are expressed as the mean ± SEM ($n = 3$). $^{*}p < 0.05$. (D) Cells were treated as described in (A) or (B). The maximum whole-cell conductance (nS) was determined when current activation reached steady-state and normalized by the cell capacitance (pF). The data are expressed as the mean ± SEM ($n = 6–10$). $^{***}p < 0.005$.

3. Results

3.1. AMPK mediates leptin-induced PTEN inactivation

Based on two key observations that leptin inhibits PTEN [18] and leptin activates AMPK [12], we questioned whether AMPK mediates leptin-induced PTEN inhibition. Because PTEN is inactivated by phosphorylation at Ser-380/Thr-382/Thr-383 residues [18], PTEN inhibition was assessed by measuring phospho-PTEN (pPTEN) levels. Western blot analysis showed that PTEN phosphorylation was obviously observed for 5 min mark after treatment with leptin (approximately a three-fold increase) and maintained thereafter in INS-1 cells, which correlated with the changes in pAMPK levels (Fig. 1A). Under this condition, the AMPK inhibitor compound C (CC) attenuated leptin-induced PTEN phosphorylation (Fig. 1B). These results were confirmed by additional pharmacologic and genetic approaches. The AMPK activator AICAR was sufficient to induce PTEN phosphorylation in the absence of leptin (Fig. 1B). In contrast, the siRNA against AMPK α-subunits (siAMPK) abolished leptin-induced PTEN phosphorylation (Fig. 1C), which is comparable to CC (Fig. 1B). These results demonstrate that leptin inhibits PTEN via the AMPK signaling pathway.

3.2. AMPK-mediated PTEN inhibition induces K_{ATP} channel trafficking

While previous studies found that PTEN inhibition leads to K_{ATP} channel activation [18], we showed that AMPK activation induces K_{ATP} channel trafficking [19]. These results lead us to hypothesize that AMPK-mediated PTEN inhibition leads to K_{ATP} channel activation via trafficking to the plasma membrane. We first examined the relationship between pPTEN and surface K_{ATP} channel levels in INS-cells and observed that the surface levels of K_{ATP} channels well correlated with pPTEN levels (Fig. 1A–C).

We then investigated whether PTEN inhibition causes K_{ATP} channel trafficking. Because PTEN has both lipid and protein phosphatase activity [21], we used four PTEN constructs in following experiments: wild-type PTEN ($PTEN^{WT}$), both phosphatase activity-defective mutant ($PTEN^{C124S}$), lipid phosphatase dead mutant ($PTEN^{G129E}$), and protein phosphatase dead mutant ($PTEN^{Y138L}$) [22]. $PTEN^{C124S}$ directly increased the surface levels (approximately a 2.5-fold) and currents of K_{ATP} channels (from 1.08 ± 0.51 nS/pF in $PTEN^{WT}$ to 4.23 ± 0.84 nS/pF in $PTEN^{C124S}$) in INS-1 cells without AMPK activation (Fig. 2A and D). These results were comparable to leptin effect on K_{ATP} channel trafficking and activity (Fig. 2A and D). PTEN inhibition by leptin or $PTEN^{C124S}$ was validated by

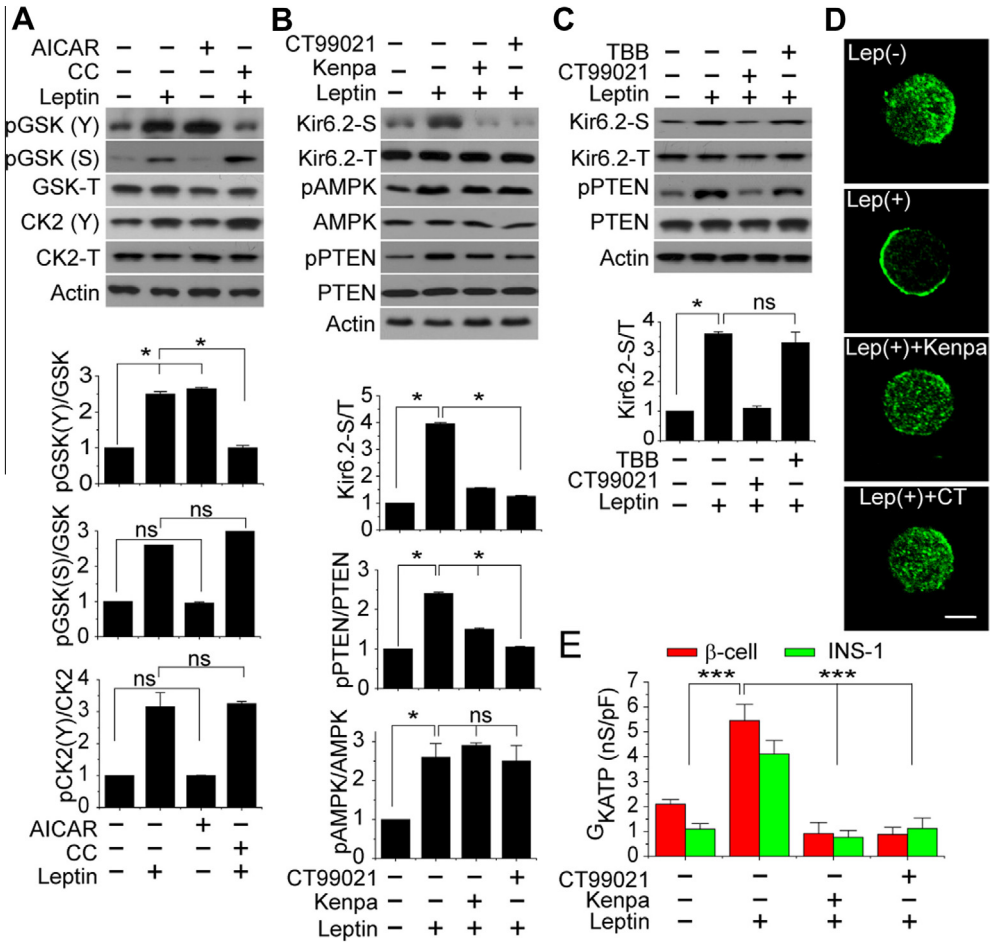

Fig. 3. GSK3β mediates AMPK-dependent PTEN inactivation and K_{ATP} channel trafficking. (A–C) INS-1 cells were treated with 10 nM leptin and/or 10 μM CC, 250 μM AICAR, 10 μM CT99021, 10 μM Kenpaullone (Kenpa), or 25 μM TBB for 30 min prior to further analyses. The relative ratios for the indicated molecules were plotted based on the quantification of the band intensities. The data are expressed as the mean ± SEM (n = 3). (D) Isolated primary pancreatic β-cells were treated with 10 nM leptin and/or 10 μM CT99021 or 10 μM Kenpaullone (Kenpa) for 30 min prior to immunofluorescence imaging using anti-Kir6.2 antibody. (E) INS-1 or primary pancreatic β-cells were treated as described in (B) and whole-cell patch clamp analysis was performed. The data are expressed as the mean ± SEM (n = 7–10). $^{***}p < 0.005$.

determining increased pAKT levels (Fig. 2A). We also found that $PTEN^{WT}$ overexpression suppressed the effect of leptin on K_{ATP} channel trafficking and activity (Fig. 2B and D). Further analyses using PTEN mutants revealed that only $PTEN^{C124S}$ elevated the surface levels of K_{ATP} channel (Fig. 2C), indicating that both protein and lipid phosphatase activities of PTEN are crucial for regulation of K_{ATP} channel trafficking and activity.

3.3. *GSK3β mediates AMPK-dependent PTEN inactivation and K_{ATP} channel trafficking*

It has been known that leptin induces PTEN phosphorylation via either glycogen synthase kinase 3β (GSK3β) or casein kinase 2 (CK2) [23], which suggests that these kinases are functionally associated with AMPK. We first examined the effect of leptin on GSK3β or CK2 activity in INS-1 cells. Leptin induced approximately a 2.5-fold increase of pGSK3β at Ser-9 (inhibitory) and Tyr-216 (activating) and a three-fold increase of pCK2 at Tyr-255 (activating) (Fig. 3A). Because GSK3β becomes active when it is concurrently phosphorylated at the Ser-9 and Tyr-216 residues [23], our results indicate that leptin concomitantly activates GSK3β and CK2. We then examined whether AMPK mediates leptin-induced activation of GSK3β or CK2 in INS-1 cells. CC suppressed Tyr-216 phosphorylation of GSK3β in leptin-treated cells, but did not affect Ser-9 phosphorylation (Fig. 3A). Conversely, AICAR increased Tyr-216 phosphorylation of GSK3β without leptin, but did not affect Ser-9 phosphorylation (Fig. 3A). In contrast, CC did not abolish leptin-induced CK2 phosphorylation and AICAR failed to increase CK2 phosphorylation (Fig. 3A). Therefore, these results indicate that AMPK mediates leptin-induced GSK3β activation, but not CK2. However, given that AMPK is a serine/threonine kinase, AMPK seems not to directly activate GSK3β.

We then investigated whether GSK3β mediates leptin-induced PTEN inactivation and K_{ATP} channel trafficking in INS-1 cells. The GSK3β inhibitors CT99021 and Kenpaullone reduced the effect of leptin on PTEN phosphorylation and K_{ATP} channel trafficking (Fig. 3B). In contrast, the CK2 inhibitor TBB did not affect leptin action on PTEN phosphorylation and K_{ATP} channel trafficking (Fig. 3C). The potent action of GSK3β on K_{ATP} channel trafficking was also investigated by immunofluorescence analysis using anti-Kir6.2 antibody. In primary pancreatic β-cells, leptin enhanced Kir6.2 signals at the cell periphery while reducing signals in the intracellular compartment (Fig. 3D). Under this condition, GSK3β inhibitors abolished leptin-induced K_{ATP} channel trafficking (Fig. 3D). These results were further validated by whole-cell patch clamp analysis. Leptin significantly increased K_{ATP} channel conductance both in β-cells (from 2.10 ± 0.17 nS/pF to 5.45 ± 0.65 nS/pF in leptin) and INS-1 cells (from 1.12 ± 0.22 nS/pF to 4.11 ± 0.54 nS/pF in leptin). The GSK3β inhibitors abrogated the effect of leptin on K_{ATP} channel conductance in both INS-1 (0.92 ± 0.44 nS/pF in

Kenpa and 0.88 ± 0.29 nS/pF in CT99021) and primary pancreatic β-cells (0.77 ± 0.27 nS/pF in Kenpa and 1.12 ± 0.42 nS/pF in CT99021) (Fig. 3E). Therefore, our results demonstrate that leptin-mediated AMPK/GSK3β signaling inactivates PTEN and thereby increases K_{ATP} channel trafficking and activity.

4. Discussion

In this study, we identified the signaling components connecting leptin to K_{ATP} channel trafficking in pancreatic β-cells: leptin inhibits PTEN via AMPK-mediated GSK3β activation and this leads to K_{ATP} channel trafficking. Pharmacologic or genetic interventions of AMPK, GSK3β, or PTEN abrogated leptin-induced K_{ATP} channel trafficking. Our results indicate that the improper function of leptin signaling constituents leads to aberrant insulin secretion in pancreatic β-cells. Our findings contribute to a much deeper understanding of the roles of adipoinsular axis in the development of obesity-associated diabetes.

Along with our previous work [12], the present study expands our knowledge regarding the leptin signaling pathways leading to K_{ATP} channel trafficking in pancreatic β-cells. To sum them all, leptin signals through TRPC4 channels to induce CaMKKβ-dependent AMPK activation, and in turn, GSK3β-mediated PTEN inhibition, which eventually causes K_{ATP} channel trafficking. We previously showed that leptin activates TRPC4 via phosphoinositide 3-kinase (PI3K) in pancreatic β-cells [12]. Given that PTEN inhibition relieves its suppressive effect on the PI3K signaling pathway [24], leptin-mediated PTEN inhibition can enable to exhibit sustained activation of TRPC4 and AMPK, creating a positive feedback loop. In fact, the activity of AMPK and the surface expression of K_{ATP} channels are observed after 5 min of leptin treatment and persisted for at least 1 h (Fig. 1A), implying that positive feedback loop can make leptin inhibition of insulin secretion more robust. However, it is still unsolved that the role of protein phosphatase activity of PTEN in regulation of K_{ATP} channel trafficking.

Our discovery may have other important clinical implications in the sense that PTEN exerts tumor suppressive activity [22]. Mice with pancreatic-specific deletion of leptin receptor showed β-cell dysfunction due to increased mass of pancreatic β-cells and elevated levels of $p70^{S6K}$, a target of AKT-mammalian target of rapamycin complex 1 (mTORC1) signaling [9], which reflects that leptin-mediated PTEN inhibition plays an important role in regulating β-cell mass. In contrast, selective deletion of PTEN in pancreatic β-cells elevates cell proliferation and reduces cell death [16]. These results show that an appropriate range of PTEN activity should be delicately regulated for maintaining the normal functions of pancreatic β-cells. Therefore, the current work may provide a conceptual framework for a deep understanding of the effect of leptin signaling pathways on pancreatic β-cell physiology.

AMPK inhibits AKT-mTORC1 signaling by phosphorylating tuberous sclerosis 2 (TSC2) [25] or Raptor, a component of mTORC1 complex [26,27]. Interestingly, our data raise the possibility that AMPK has also the ability to activate AKT-mTORC1 signaling through PTEN inhibition, which counterbalances AMPK-mediated mTORC1 inhibition. Considering the previous findings that prolonged AMPK activation induce apoptosis in pancreatic β-cells [28,29], our findings may provide an exemplar of a balancing mechanism that regulates signal strength and phenotypic outputs.

In summary, we here demonstrate that leptin induces K_{ATP} channel trafficking via AMPK/GSK3β-mediated PTEN inhibition in pancreatic β-cells. Our findings assist in gaining a broader perspective on leptin action on pancreatic β-cell physiology and impart crucial epistemic insight into the epidemiologic association between obesity and diabetes.

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