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ARHGAP21 prevents abnormal insulin release through actin rearrangement in pancreatic islets from neonatal mice

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ABSTRACT

Aims: ARHGAP21 is a Rho GTPase-activating protein (RhoGAP) that associates with many proteins and modulates several cellular functions, including actin cytoskeleton rearrangement in different tissues. However, it is unknown whether ARHGAP21 is expressed in pancreatic beta cells and its function in these cells. Herein, we assess the participation of ARHGAP21 in insulin secretion.
Main methods: Neonatal mice were treated with anti-sense oligonucleotide against ARHGAP21 (AS) for 2 days, resulting in a reduction of the protein's expression of about 60% in the islets. F-actin depolimerization, insulin secretion, mRNA level of genes involved in insulin secretion, maturation and proliferation were evaluated in islets from both control and AS-treated mice.
Key findings: ARHGAP21 co-localized with actin in MIN6 beta cells and with insulin in neonatal pancreatic islets. F-actin was reduced in AS-islets, as judged by lower phalloidin intensity. Insulin secretion was increased in islets from AS-treated mice, however no differences were observed in the GSIS (glucose-stimulated insulin secretion). In these islets, the pERK1/2 was increased, as well as the gene expressions of VAMP2 and SNAP25, proteins that are present in the secretory machinery. Maturation and cell proliferation were not affected in islets from AS-treated mice.
Significance: In conclusion, our data show, for the first time, that ARHGAP21 is expressed and participates in the secretory process of pancreatic beta cells. Its effect is probably via pERK1/2, which modulates the rearrangement of the cytoskeleton. ARHGAP21 also controls the expression of genes that encodes proteins of the secretory machinery.

Introduction

Glucose is the master regulator of insulin secretion [19] and its metabolism produces several signals, including increased cytosolic calcium content, which promotes actin cytoskeleton rearrangement and leads to the translocation and docking of the insulin-containing granules, resulting in insulin release [21,32]. In this context, the actin cytoskeleton plays a crucial role in glucose-stimulated insulin secretion (GSIS). It is localized near the plasma membrane and forms a physical barrier that prevents the approximation of insulin granules to the membrane. For effective insulin secretion, a transient rearrangement of this actin cytoskeleton is necessary to allow the movement of insulin-containing granules and their docking at the plasma membrane [14,15,32]. Calcium ions are an important regulator of this process, as increases in cytosolic calcium concentrations are associated with the activation of several proteins that regulate actin dynamics [12,13,19,24]. This process is crucial for a normal insulin secretion, and defects in this step may promote abnormal insulin release and impairments in glucose homeostasis that could culminate in Type 2 Diabetes [8]. In this scenario, identifying proteins that regulate the actin dynamics in the pancreatic beta cell could be an efficient strategy to treat Type 2 Diabetes by restoring GSIS. Here, we tested whether ARHGAP21, a protein involved in actin dynamics in several cell types, could be a regulator of this process in pancreatic beta cells.

ARHGAP21 is a member of the RhoGAP family and has been described in many cell types. It associates with several proteins, such as FAK [4], PKCζ [5], α catenin, β arrestin 1, Cdc42 and ARF1 [1,4,5,9,20,29]. Due to these protein interactions and its RhoGAP activity, ARHGAP21 has been found to exert important functions including mechanical stress [5], cytoskeleton organization and cell migration [2,4,17], formation of cell–cell adherent junctions [2,29], modulation

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of retrograde trafficking of vesicles [10], differentiation [3], cell proliferation, and gene expression [17].

This interesting protein has not yet been described in pancreatic beta cells, and its possible role in GSIS is unknown. Based on the fact that ARHGAP21 has the capacity to interact with and to modulate the activities of several proteins involved in actin rearrangement, we hypothesized that ARHGAP21 could modulate actin rearrangement and, consequently, insulin secretion.

Here, we report that ARHGAP21 is expressed in pancreatic beta cells and modulates insulin release through actin rearrangement. ARHGAP21 also controls the expression of genes that encode proteins involved in the secretory machinery.

Material and methods

Reagents

Rabbit anti-ARHGAP21 polyclonal antibody, which is specific for residues 1856–1870 of the human protein sequence, was generated by Bethyl Laboratories (Montgomery, ***Texas***). The polyclonal ***Guinea*** Pig anti-insulin antibody was from Dako ***North*** America, Inc. (Carpinteria, CA, USA). The polyclonal Rabbit anti-ERK1/2 and pERK1/2 were purchased from ***Santa Cruz*** Biotechnology, Inc. (Dallas, ***Texas***, USA). The secondary antibody goat anti-Rabbit was obtained from Thermo Scientific. Chicken anti-Rabbit AlexaFluor® 488 and AlexaFluor® 568 phalloidin were acquired from Life Technologies (Foster City, CA, USA). Mounting medium for fluorescence was purchased from Vector Laboratories (Burlingame, CA, USA); and Rabbit Anti-***Guinea*** Pig IgG–TRITC antibody and routine reagents were from ***Sigma-Aldrich*** (St. Louis, MO, USA). The Fast SYBR® Green Master Mix and High-capacity cDNA Reverse Transcription Kit were acquired from Life Technologies (Foster City, CA, USA). Primers and oligonucleotides (antisense anti-ARHGAP21 and mismatch) were acquired from Sigma

Animals

Neonatal Swiss mice aged 5–6 days from the State University of Campinas animal facilities were used in the experiments. All experiments were approved by the Committee for Ethics in Animal Experimentation of the State University of Campinas (CEEA/IB/UNICAMP). The mice, together with their mothers, were maintained on a 12 h light–dark cycle at 20–21 °C with controlled humidity. The neonatal mice, when 2 days-old, received daily subcutaneous injections of 1 nmol anti-ARHGAP21 oligonucleotide (AS) or Mismatch (CTL) in Tris–EDTA buffer for 2 consecutive days. At 12 h after the last injection, the mice were killed by decapitation and the pancreas was removed to perform all experiments. The oligonucleotides utilized were as follows:

Anti-ARHGAP21: *mC*mU*mU*mU*C*C*T*C*C*T*C*T*G*T*mU*mU*mC*mC*

Mismatch: *mC*mU*mU*mU*C*T*A*C*C*T*C*A*G*T*mU*mU*mC*mC*

Immunofluorescence and confocal microscopy

Immunofluorescence of MIN6 cells was performed as described [4]. MIN6, kindly provided by Dr. Oka and Prof. J. I. Miyazaki (Tokyo, Japan), were cultured in a monolayer in RPMI 1640 medium (Sigma-Aldrich), supplemented with 10% (vol./vol.) of fetal bovine serum, penicillin, and streptomycin in a humidified atmosphere at 37 °C and 5% CO_2. For the detection of ARHGAP21 and actin, cells were cultured on coverlips, fixed in 4% paraformaldehyde-PBS for 20 min and then permeabilized with 0.5% Triton-X-100 in PBS for 10 min. The cells were blocked with 5% BSA–PBS and incubated with the primary (overnight, 4 °C; dilution 1:50) and secondary (1 h, room temperature; dilution 1:500) antibodies and with phalloidin–TRITC for 30 min. Neonatal mice pancreases were prepared for immunofluorescence [7]. Pancreases were frozen in n-hexane with liquid nitrogen and cryosectioned (6 μm). For the detection of ARHGAP21, insulin or phalloidin, the sections were fixed with acetone at −20 °C for 3 min. The sections were then fixed with 2% paraformaldehyde-PBS for 8 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min. All sections were blocked for 1 h with 5% BSA–PBS and incubated with the primary (anti-ARHGAP21 — dilution 1:50, anti-insulin — dilution 1:100 or phalloidin — dilution 1:40) and secondary (dilution 1:250) antibodies. Finally, the slides were mounted using Mounting Medium for Fluorescence, and the tissues were analyzed by confocal laser scanning with a LSM 510 (Zeiss, Welwyn Garden City, UK) mounted over an axioplan using 40× (pancreatic islet) and 60× (MIN6 cells) oil immersion objectives.

Islet isolation and insulin secretion

Pancreatic islets were isolated as described [28]. The pancreases were incubated in Hanks buffer (137 mM NaCl, 5.5 mM KCl, 4.5 mM $NaHCO_3$, 0.4 mM KH_2PO_4, 0.4 mM Na_2HPO_4, 0.8 mM $MgSO_4$, 1.5 mM $CaCl_2$, pH 7.4) containing 0.66 mg/mL of colagenase for 5 min at 37 °C. After the incubation, the solution was gently stirred to allow complete dissociation of the islets. The islets were collected and pre-incubated in groups of ten, for 1 h at 37 °C, in Krebs bicarbonate buffer (KRBB — 115 mmol/L NaCl, 5 mmol/L KCl, 2.56 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 10 mmol/L $NaHCO_3$, 15 mmol/L HEPES, pH 7.4), containing 2.8 mmol/L glucose and 0.2 g/L BSA. Subsequently, the islets were incubated again for 1 h at 37 °C in a similar buffer with 2.8, 5.6, 8.3, 11.1, 16.7 or 22.2 mmol/L of glucose. After incubation, the supernatant was collected and insulin content was measured by radioimmunoassay. The islets were used for DNA measurement. The insulin secretion was normalized by the DNA content.

DNA extraction

Groups of ten islets were incubated with 500 μL of Tris–HCl/EDTA buffer (50 mM Tris–HCl, 10 mM EDTA and 1% SDS) for 30 min at 37 °C. After incubation, 200 μL of a mix of phenol, chloroform and isoamyl alcohol (25:24:1) was added, and the islets were gently shaken for a few seconds, before centrifuging for 10 min at 16,000 *g*. Two to three hundred microliters of the upper phase, formed in the tubes, was transferred to 1.5 mL vials and a similar volume of chloroform was added. The mixture was gently shaken, before centrifuging for 3 min at 12,000 *g*. One hundred microliters of the upper phase was collected and DNA content was measured using the SybrGreen method.

Western blotting and protein expression

Pancreatic islets from CTL or AS neonatal mice were lysed with urea anti-protease/anti-phosphatase buffer (7 mM urea, 2 M thiourea, 5 mM EDTA, 1 mM sodium fluoride, 1 mM orthovanadate, 1 mM pyrophosphate and 2 mM phenylmethylsulfonyl fluoride) and the expressions of ARHGAP21 and pERK1/2 were evaluated by Western blotting, as described [28]. The intensities of the protein bands were detected using a LAS-3000 CCD camera, and quantification was performed using densitometry (ImageJ, Bethesda, USA). The densitometry values of ARHGAP21 were normalized by GAPDH band intensities, while pERK1/2 band densitometry was normalized by ERK1/2.

Quantitative real-time PCR

Pancreatic islet mRNA was extracted by RNeasy kit (Qiagen, Cat. #74,007). One microgram of purified mRNA was then used to synthesize cDNA (High-Capacity cDNA reverse transcription kit, Applied Biosystems, Foster City, CA). The primers were designed and tested against the Mus musculus genome (GenBank). The relative quantities of the target transcripts were calculated from duplicate samples ($2^{\Delta\Delta CT}$), and the data were normalized against an endogenous control, GAPDH. The genes studied were as follows; NKX6, PDX1, MAFA,

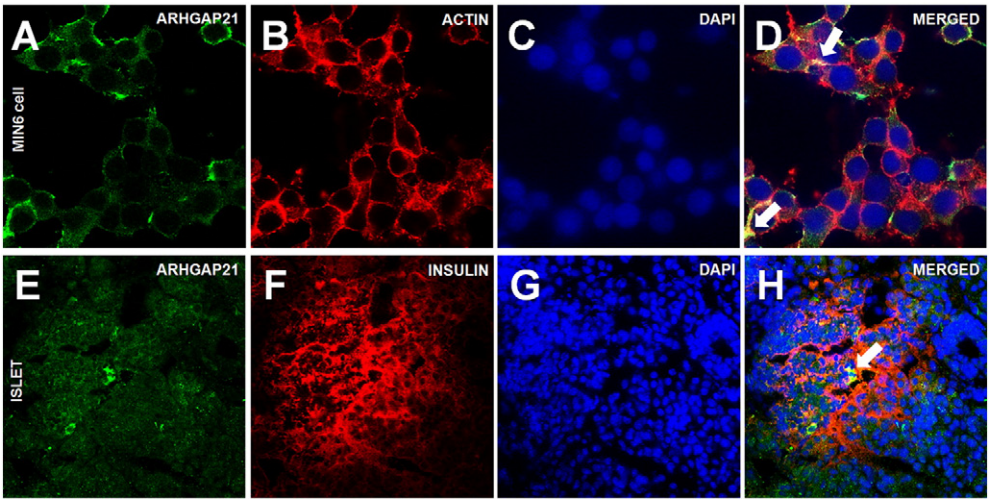

Fig. 1. ARHGAP21 localization in pancreatic beta cells. MIN6 beta cells (A–D) were fixed and stained for ARHGAP21 (A), actin (B) and DAPI (C). Images were captured using a 60× oil immersion objective. ARHGAP21 localizes predominantly in the cytoplasm (A) and co-localizes with actin (D, arrow). Neonatal mice pancreatic islets (E–H) were fixed and stained for ARHGAP21 (E), insulin (F) and DAPI (G). Images were captured using a 40× oil immersion objective. ARHGAP21 is present predominantly in the cytoplasm of beta cells (E) and co-localizes with insulin (F, arrow). Images are representative of 3 different experiments.

HNF4α, INS1, VAMP2, SYNATXIN 1A, SNAP25, CYCLIN D2 and CDK4. The primer sequences are available in the. Relative quantification was performed using the Step-one real-time PCR system (Applied Biosystems).

Statistical analysis

Data are expressed as means ± SEM. Statistical analyzes were performed using Student's t-test. $P < 0.05$ was considered to be statistically significant.

Results

Expression and localization of ARHGAP21 in MIN6 beta cells and neonatal mice pancreatic islets

We first evaluated whether ARHGAP21 is expressed in pancreatic beta cells and its localization. For this, we performed immunofluorescent staining in MIN6 beta cells and in neonatal islets. ARHGAP21 was expressed in MIN6 beta cells and neonatal islets and; in both tissues,

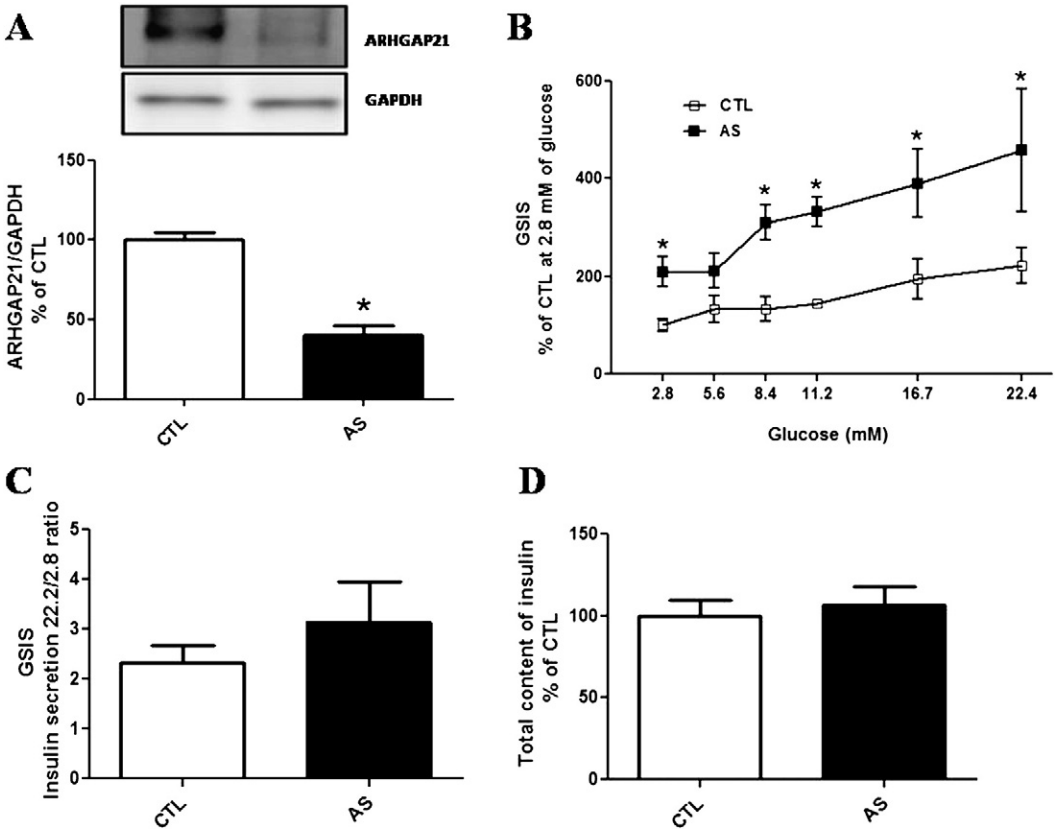

Fig. 2. ARHGAP21 knockdown and insulin secretion. Neonatal Swiss mice received daily subcutaneous injections of 1 nmol/L of anti-ARHGAP21 (AS) or Mismatch (CTL) oligonucleotides for 2 consecutive days. ARHGAP21 expression was evaluated by Western blotting (A). Pancreatic islets from CTL and ARHGAP21 knockdown (AS) mice were incubated with 2.8, 5.6, 8.3, 11.1, 16.7 or 22.2 mmol/L of glucose, and insulin secretion was assessed by radioimmunoassay (B). GSIS (C). Insulin content (D). N = 6; *$p < 0.05$ vs. CTL. Values are the means ± SEM.

its localization was predominantly in the cytoplasm (Fig. 1A and E). The co-localization of ARHGAP21 with actin in MIN6 beta cells (Fig. 1D, arrow) and with insulin in pancreatic islets (Fig. 1H, arrow) is illustrated. These results allowed us to hypothesize that ARHGAP21 could modulate insulin secretion.

ARHGAP21 inhibits basal insulin secretion

To test our hypothesis that ARHGAP21 could modulate insulin secretion we evaluated insulin secretion in an experimental model of islets from control and AS-mice, presenting a reduction of about 60% in ARHGAP21 expression (Fig. 2A). We observed that pancreatic islets from AS mice secreted significantly more insulin in response to sub- (2.8 mmol/L) and stimulatory (8.3, 11.1, 16.7 and 22.2 mmol/L) glucose concentrations (Fig. 2B). However, the increase in insulin secretion under stimulatory conditions resulted from the higher insulin released in sub-stimulatory glucose condition (Fig. 2B). The total insulin content did not differ between the groups (Fig. 2D). Based on these results, we conclude that ARHGAP21 seems to be important for controlling insulin release, under basal glucose concentrations, and that it did not interfere in the expression of insulin.

ARHGAP21 modulates actin dynamics

It is known that insulin release under sub-stimulatory glucose conditions is prevented by F-actin [15,21,32]. Thus, we evaluated the presence of F-actin in islets from mice in which ARHGAP21 was transiently reduced. We stained the islets with phalloidin, a compound that has a higher affinity for the F-actin state. The staining intensity was lower in AS, compared to control islets (Fig. 3A and B). In an attempt to find possible components associated with the reduced F-actin state in AS islets, we assessed ERK1/2 phosphorylation, which is important for actin depolymerization in pancreatic beta cells [6,30]. In fact, the pERK1/2 content was significantly higher in the islets from ARHGAP21-knockdown mice, compared with control mice (Fig. 3C). This result indicates that ARHGAP21 may be important for the maintenance of F-actin under sub-stimulatory glucose conditions and that this regulation probably occurs via pERK1/2.

ARHGAP21 modulates the expression of genes involved in the insulin secretory machinery

It is known that the actin cytoskeleton and proteins of the secretory machinery interact to regulate insulin release [15]. As ARHGAP21 modulates insulin secretion by preventing F-actin depolymerization, we hypothesized that ARHGAP21 could modulate the expression of genes that encode proteins of the secretory machinery. For this, we assessed the gene expression of proteins involved in the secretory machinery of insulin granule, such as VAMP2, SNAP25 and SYNTAXIN 1A (Fig. 4). In islets with transiently-reduced expression of ARHGAP21, we noted increased expressions of the VAMP2 and SNAP25 genes, indicating that ARHGAP21 could modulate insulin secretion through the regulation of genes that encode proteins of the secretory machinery.

As the secretion of insulin in islets from AS-treated neonatal mice was higher, and since the increase in insulin secretion may result from augmented proliferation and maturation of islets [23,25,31], we analyzed the effects of ARHGAP21 on these two parameters. For this, we assessed the mRNA levels of genes involved in islet beta cell maturation (NKX6.1, PDX-1, MAFA, HNF4α and INS1) (Fig. 5A), and proliferation (CYCLIN D2 and CDK4) (Fig. 5B). No differences in the mRNA levels of these genes, compared with the control, were observed. Our results suggest that ARHGAP21 modulates insulin secretion at sub-stimulatory

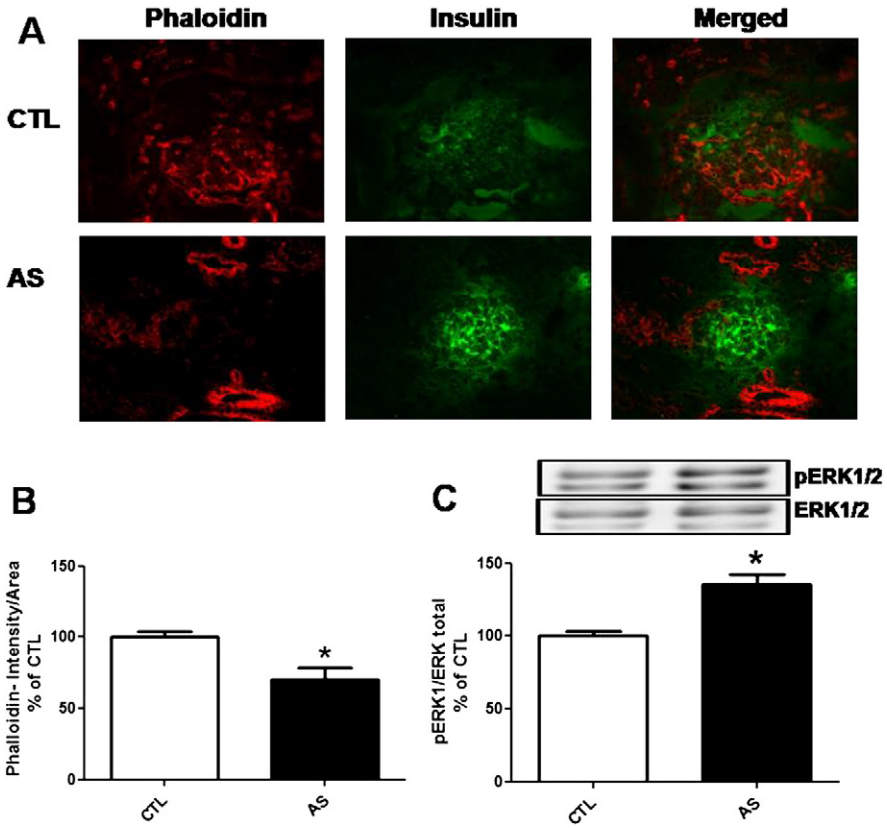

Fig. 3. F-actin and ERK1/2 phosphorylation. F-actin was determined by measuring the intensity of phalloidin staining (A and B). Images were captured using a 40× objective. The quantification of the intensity of phalloidin staining was performed using densitometry (ImageJ, Bethesda, USA). ERK1/2 phosphorylation was assessed by Western blotting (C). Results were expressed as % of the control. N = 4; *p < 0.05 vs. CTL. Values are the means ± SEM.

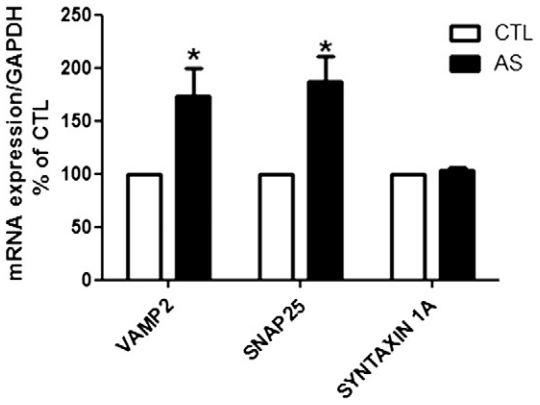

Fig. 4. Expression of genes involved in secretory machinery in pancreatic islet. The gene expression of VAMP2, SNAP25, and SYNTAXIN 1A were assessed by real-time PCR. N = 4–8; *p < 0.05 vs. CTL Values are the means ± SEM.

concentrations of glucose by preventing F-actin depolymerization. ARHGAP21 also controls the expression of some genes that encode proteins of the secretory machinery, without affecting cell maturation and proliferation.

Discussion

It is known that ARHGAP21 is expressed in several cell types where it exerts important functions [4,5,9,20,2,10,11,17]. Here, we show, for the first time, that ARHGAP21 is expressed in MIN6 beta cells and pancreatic islets from neonatal Swiss mice and that it is involved in the mechanism of insulin secretion.

ARHGAP21 preferentially localizes in the cytoplasm of MIN6 beta cells and pancreatic islets cells from neonatal mice. In contrast, in cardiomyocytes, ARHGAP21 is localized in the nucleus [5], whereas in normal human fibroblast cell lines, HeLa cells, human glioblastoma cells and human prostate cancer cells, its localization is perinuclear and nuclear [2,4,17].

In our experimental model, in which expression of ARHGAP21 is transiently reduced (−60%), we found that GSIS was increased, mostly in sub-stimulatory glucose concentrations, indicating that ARHGAP21 may be important for preventing insulin release under non-stimulatory glucose conditions. This modulation probably occurs via actin dynamics.

ARHGAP21 co-localizes with actin in MIN6 beta cells. Therefore, it seems to be important for the maintenance of the F-actin state, based on the fact that phalloidin staining was reduced in AS-islets. These results suggest that, under sub-stimulatory glucose concentrations, ARHGAP21 can inhibit the depolymerization of F-actin, contributing to insulin secretion control at low glucose concentrations. It is known that F-actin filaments form a barrier underneath the plasma membrane, preventing the approach of the insulin granule to the membrane, and consequently impairing insulin release. Thus, actin reorganization is necessary for granule mobilization and insulin secretion [15,32]. Glucose promotes a transient depolymerization of F-actin and consequent insulin release (Thurmond, 2013). Glucose metabolism generates signals, including an increase in cytosolic calcium, which activates regulatory proteins involved in actin rearrangement, such as ERK1/2 [15,18]. In our model, we observed that knockdown of ARHGAP21 increased ERK1/2 phosphorylation, indicating that ARHGAP21 may be involved in actin dynamics, probably via regulation of ERK1/2 activity.

The increase in insulin secretion at sub-stimulatory glucose concentrations was also associated with the depletion of other proteins that regulate actin dynamics, such as Cool-1/βpix [16], Cav-1 [22] and FAK [26,27]. Thus, our results strongly suggest that, in the presence of non-stimulatory glucose concentrations, ARHGAP21 may inhibit insulin secretion, mostly through the organization of the actin cytoskeleton.

In islets in which ARHGAP21 was reduced, we observed an increase in the expression of VAMP2 and SNAP25, genes that belong to the SNARE family and participate in insulin release [15]. These data indicate that ARHGAP21 can also modulate insulin secretion through the regulation of gene expression. The effect of ARHGAP21 on gene expression has also been described in prostate adenocarcinoma PC3 cells, in which the depletion of ARHGAP21 modulates the expression of genes involved in proliferation, cytoskeleton organization, and the canonical pathway of endothelin-1 [17]. In addition, the reduction in ARHGAP21 expression increased cell proliferation in PC3 cells [17]. However, we did not observe any alteration in the expression of genes involved in cell maturation (NKX6.1, PDX-1, MAFA, HNF4α and INS1) or proliferation (CYCLIN D2 and CDK4) when ARHGAP21 was transiently reduced in mice. One explanation for this discrepancy could be due to the use of (a) in vivo experiments in contrast to cell line assays, (b) pancreatic islets in contrast to prostate adenocarcinoma cells line and (c) differences in experimental conditions.

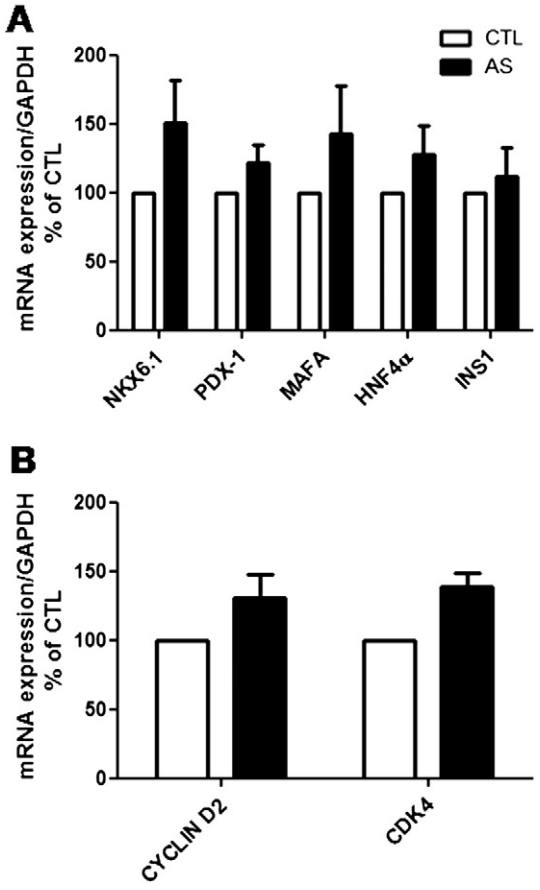

Fig. 5. Expression of genes involved in maturation and proliferation in pancreatic islets. NKX6.1, PDX-1, MAFA, HNF4α and INS1 involved in maturation (A); and CYCLIN D2 and CDK4 involved in proliferation (B) were assessed by real-time PCR. N = 4–8; values are the means ± SEM.

Conclusions

In conclusion, we show that ARHGAP21 is expressed in pancreatic beta cells and in a mouse beta cell lineage. Our results suggest that ARHGAP21 prevents insulin release at sub-threshold glucose concentrations in neonatal pancreatic islets. This modulation may be mediated by the depolymerization of F-actin. ARHGAP21 also modulates the expression of genes that encode proteins involved in secretory machinery, such as VAMP2 and SNAP25.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.lfs.2015.01.041.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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