Overexpression of *ZAC* Impairs Glucose Stimulated Insulin Translation and Secretion in Clonal Pancreatic Beta-Cells

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Abstract

Background—*ZAC* (Zinc finger protein that regulates Apoptosis and Cell-cycle arrest) is a candidate gene for transient neonatal diabetes mellitus (TNDM). This condition involves severe insulin deficiency at birth that reverses over weeks or months but may relapse with diabetes recurring in later life. *ZAC* overexpression in transgenic mice has previously been shown to result in complex changes in both beta-cell mass and possibly function. The present study therefore aimed to examine the role of *ZAC* in beta-cell function *in vitro*, independently of the confounder of a reduced beta-cell mass at birth.

Methods—Overexpression of *ZAC* was achieved though the tetracycline-regulatable system in the beta-cell line, INS-1.

Results—We found that *ZAC* overexpression exerted no significant effect on proliferation in this transformed cell line at any of the glucose concentrations examined. By contrast, glucose-stimulated insulin secretion was impaired through a mechanism downstream of cytosolic Ca^{2+} increases, which was unaffected. Furthermore, glucose-stimulated proinsulin biosynthesis was inhibited despite an increase in insulin transcript levels. Finally, we found that glucose downregulated *ZAC* expression in both INS-1 cells and primary mouse islets.

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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Conclusions—These results indicate that *ZAC* is a negative regulator of the acute stimulatory effects of glucose on beta-cells, and provide a possible explanation for both insulin deficiency in the neonate, and the later relapse of diabetes, in TNDM cases.

Keywords

ZAC; Beta-cell; Insulin; Translation; Secretion

Introduction

Decreased beta-cell mass and impaired beta-cell function are involved in all types of diabetes, but the molecular mechanisms underlying these deficiencies are not fully understood. A complete understanding of the molecular basis of beta-cell growth and function in normal and pathological conditions is essential for the cure of this disease. Studies on monogenetic diabetes have identified genes that play important roles in beta-cells, such as the genes responsible for MODY (Maturity-Onset Diabetes in the Young, types 1 to 8) and neonatal diabetes mellitus (NDM). One NDM gene whose disease-causing mechanism has received very little attention to date is *ZAC*, a gene responsible for Transient Neonatal Diabetes Mellitus (TNDM). The purpose of this paper is to examine the effect of *ZAC* overexpression on beta-cell function.

TNDM is a rare disorder involving severe but temporary insulin deficiency at birth. After a remission phase, diabetes relapses in 50% to 60% of cases at age 4-25 years [1]. Insulin secretion in response to glucose stimulation is impaired with no evidence of insulin resistance [2]. Most TNDM are due to two-fold increased expression of an imprinted gene with exclusive paternal expression at Chr 6q24 as a result of Chr 6 paternal uniparental isodisomy [1], paternal duplication at 6q24 [3] or methylation defect on the maternal allele [4]. Two imprinted genes, *ZAC* (zinc finger protein that regulates apoptosis and cell cycle arrest, also called *PLAGL1*, pleiomorphic adenoma gene-like 1) and *HYMAI* (hydatidiform mole-associated and imprinted transcript) map to this region [4]. HYMAI encodes an untranslated mRNA of unknown function. ZAC is a well identified transcription factor, studied to date mostly as a tumour-suppressor gene.

ZAC encodes a zinc finger protein expressed in many tissues and can induce apoptosis and growth arrest [5]. Loss or decreased expression of *ZAC* has been found in many different tumours [6–14].

A PAC transgenic construct carrying the TNDM locus, containing both *ZAC* and *HYMAI,* recapitulates the neonatal hyperglycemia and impaired adult glucose tolerance when paternally inherited, with impaired beta-cell development [15]. However, the phenotype was much milder than in the human disease and effects on development vs. mature function could not be dissected. The multiple functions of *ZAC* suggest that it may be involved in both. In the present study, we examine the effect of its subtle overexpression on beta-cell function *in vitro* in a beta-cell line, independently of the confounder of a reduced beta-cell mass at birth.

Materials and Methods

Cell culture

The rat INS-1 beta-cell line was cultured in RPMI1640 (Invitrogen, Burlington, ON, Canada) containing 10% FBS, 10 mmol/l HEPES, 1 mmol/l sodium pyruvate, 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μmol/l β-mercaptoethanol, and 11.1 mM glucose.

Reverse transcriptase-PCR

Expression of *ZAC* orthologs in different cell lines and mouse islets were examined by RT-PCR. Equal amounts of RNA was used in each reverse transcription reaction. Forward primer sequence for the PCR is CCTGCTGGACCACCTCAAGTC and the reverse primer sequence is CGAGTGAGGTGGTCTTTGCG. *18S* was used as reference standards.

Establishment of doxycycline- (Dox) inducible ZAC expression in INS-1 cells

The INS-1 derived INS-R9 clone, which contains the reverse tetracycline controlled transactivator (rt-TA), was a gift from Dr. Patrick B Iynedjian (Geneva, Switzerland) and Dr. Marc Prentki (Montreal, QC Canada). It was stably transfected with pTRE2-*ZAC*-pur, a plasmid encoding human ZAC driven by a minimal cytomegalovirus promoter placed under control of the tetracycline operator [16], constructed by subcloning the *ZAC* cDNA into the expression vector pTRE2-pur (Clontech, Mountain View, CA, U.S.A.). Puromycin (200 ng/ml, Clontech, Mountain View, CA, U.S.A.) was added into the medium for selection. After 3 weeks, resistant clones were picked and maintained in 100 ng/ml puromycin and 100 μg/ml G418 (Invitrogen, Burlington, ON Canada).

Competitive RT-PCR

To compare induced *ZAC* expression with endogenous *Lot1* (rat ortholog of *ZAC*) expression in INS-R9-2 cells, we used RT-PCR with primers corresponding to sequences identical in *ZAC* and *Lot1*, and produce the same length of product, followed by digestion with NdeI which cuts *ZAC* but not *Lot1.* After running on 1% agarose gel, band intensities were measured by densitometry. Then, to define the expression of *ZAC* in INS-R9-2 cells in reference to physiologic *Lot1* expression in beta-cells, an equal mixture of RNA from purified rat islets and INS-R9-2 cells treated with various doses of Dox was subjected to competitive PCR as above.

Real-Time PCR

Total RNA was isolated using RNeasy Plus Mini Kit (Qiagen, Mississauga, ON Canada). Reverse transcription was carried out with SuperScipt™ II Reverse Transcriptase (Invitrogen, Burlington, ON Canada). cDNA was mixed with the TaqMan universal PCR Master Mix (Applied Biosystems, Foster City, CA U.S.A.) and the TaqMan Gene Expression Assays probes, Hs00414677_m1 for *ZAC*, Mm00494251_m1 for mouse *Zac1*, Rn00688897_m1 for rat *Lot1*, RN01774648_G1 for *rIns2*, and 4352930E for *18S* (Applied Biosystems, Foster City, CA U.S.A.). The cycle count of *18S* was used as reference for standardization after 1:10,000 dilution. In all experiments, the samples being compared were

obtained, purified, stored and assayed side-by-side under identical conditions with approximately equal amounts of input RNA by spectrophotometry.

[^{3}H] thymidine incorporation

INS-R9-2 cells were cultured on 12-well plates (1.5 x 10^5 cells per well) in the presence or absence of Dox (1000 ng/ml) for 48 or 96 h. Then medium was replaced by serum-free RPMI 1640 with 2.5 mM glucose and 0.1% BSA for 24 h with or without Dox, and then incubated for a further 24 h in RPMI 1640, 0.1% BSA at different glucose concentrations (2.5-25 mM glucose) with or without Dox. [^{3}H]thymidine was added in the last 4 h of incubation. After washing, precipitation with 5% TCA and lysis with 0.5 N NaOH/0.5% SDS, 50 µl of lysate were quantified by scintillation counting.

Caspase 3 activity assay

INS-R9-2 cells were cultured in medium containing 1% FBS and 11.1 or 25 mM glucose in the presence or absence of Dox (1000 ng/ml) for 3 or 5 days. Cells were lysed and caspase 3 activity was measured by using the CaspACE™ Assay System, Colorimetric (Promega, G7220, San Luis Obispo, CA USA) and normalized by total protein.

Insulin secretion and insulin content

INS-R9-2 cells were cultured in 12-well plates (2.2 x 10^5 cells per well) in standard medium in the presence or absence of Dox for 36 h. Then cells were equilibrated in medium containing 2.5 mM glucose with or without Dox for 16 h. After two washes with Krebs-Ringer bicarbonate HEPES buffer (KRBH, 140 mmol/l NaCl, 3.6 mmol/l KCl, 0.5 mmol/l NaH$_2$PO4, 0.5 mmol/l MgSO4, 1.5 mmol/l CaCl$_2$, 2 mmol/l NaHCO$_3$, 10 mmol/l HEPES, pH 7.4) containing 2.5 mM glucose and 0.1% BSA, cells were stimulated with glucose for 1 h, or 20 mM KCl in 2.5 mM glucose for 30 min. KRBH buffer was collected and insulin was detected using a rat Insulin ELISA (Mercodia, Winston Salem, NC U.S.A.). Insulin in cells was extracted by acid-ethanol (75% ethanol: 1.5% HCL: 23.5% H$_2$O) as previously described [17].

Intracellular [Ca^{2+}] measurements

Cells were plated on glass coverslips and incubated in standard media in the presence or absence of doxycycline (1000 ng/ml) for 36 hrs, then 'starved' in media containing 2.5 mM glucose with or without Dox for 16 h. Cells were loaded with 200 nM Fura-red (Invitrogen) in KRBH containing 2.5 mM Glucose with or without Dox at 37 °C for 45 minutes before imaging. Cells were perifused with KRBH + 0.1% BSA and 25 mM glucose for 30 min at 1 ml/min, followed by 20 mM KCl for 3 min to confirm the maximum response of the cells. Where K$^+$ was increased, Na$^+$ concentration was reduced to maintain osmolarity. Cells were excited at 480/440 nm, and images captured, using an Olympus IX-81 microscope coupled to an F-view camera and captured using Cell^R software (Olympus, Hertfordshire, U.K.) with a 40 × oil objective [18]. Data are expressed as the ratio of the fluorescence emission at 440/480 nm. The 'peak' first minute of the response to K$^+$ correlates with the secretory capability of the cell and was calculated from the onset of the response using OriginPro7.5 software (OriginLab, Northampton, MA). The "area under the curve" (AUC) for the

measured Ca^{2+} increases was calculated using OriginPro7.5 software (OriginLab, Northampton, MA) and statistical significance was tested by ANOVA.

Proinsulin biosynthesis

INS-R9-2 cells were cultured in 6-well plates (2.7 x 10^5) in standard medium in the presence or absence of Dox (1000 ng/ml) for 96 h, then washed and incubated in KRBH with 0.5 mM glucose for 2 hours. Cells were then incubated in KRBH containing 0.5 mM or 16.7 mM glucose for 10 min, following which ^{35}S Methionine/Cysteine (PerkinElmer, Montreal, QC Canada) were added for 30 min. Cells were washed and lysed with ice-cold lysis buffer (100 mM NaCl, 1% Triton X-100, 0.2% sodium deoxycholate, 0.1% SDS, 10 mM EDTA, and 25 mM Tris, pH 7.4), containing proteinase inhibitors (Roche Applied Science). Total protein synthesis was measured by TCA precipitation and lysate, normalized for total protein was immunoprecipitated with proinsulin antibody (Novus, Littleton, CO USA) and protein A agarose (Stratagene), and eluted in 3% SDS (wt/vol), 1.5% mercaptoethanol (vol/vol), 7.5% glycerol (wt/vol), 0.0125% Coomassie blue G-250 (Serva), 37.5 mM Tris/HCl (pH 7.0). Labeled proinsulin was detected by Tricine-SDS PAGE [19], fluorography and densitometry.

Mouse islet isolation

To study the effects of glucose on *Zac1* expression, pancreas from C57BL/6 mice (6~9 weeks) was excised after duct injection of collagenase, and digested for 20-25 minutes at 37°C. Islets were hand picked 3 times and recovered in RPMI 1640, containing 10% FBS, 10 mmol/l HEPES, 1 mmol/l sodium pyruvate, 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μmol/l β-mercaptoethanol, and 11.1 mM glucose, overnight. Then medium was changed to 3 mM glucose for 24 h, followed by incubation with different glucose concentration, 3 mM and 11.1 mM for 24 h. RNA was extracted with RNeasy plus mini kit (Qiagen).

All data were expressed as mean ± SEM. Student's t-test was used to evaluate significance of difference between two groups. For Multiple comparisons we used ANOVA followed by Tukey's test. A value of P < 0.05 was considered statistically significant.

Results

Inducible ZAC expression in an INS-1 cell clone

We show that *Zac1* is expressed in different beta-cell lines and in adult mouse islet (Fig. 1A). The INS-R9 cell line, expressing the reverse tetracycline-dependent transactivator (rt-TA), was stably transfected with a plasmid encoding *ZAC* under the control of the tetracycline operator. Clones were selected for Dox-induced *ZAC* expression and glucose stimulated insulin secretion. One clone designated INS-R9-2 showed the highest induced expression (Fig. 1B). Maximal induction, ~9 fold, was achieved with 1000 ng/ml of Dox (Fig. 1C).

Lot1 expression in INS-R9-2 cells and in rat islets

Because human TNDM results from *ZAC* overexpression at only twice the physiologic level, we sought a Dox dose that approximated a level not much higher than physiological.

Towards this goal, we first compared induced *ZAC* expression (48 h) with endogenous *Lot1* expression in INS-R9-2 cells by competitive PCR. *Lot1* is expressed in this transformed cell line at very low levels (Fig. 2A, upper band with 512 bp in lane 2), comparable to the low-level leaky expression of the human construct (Fig. 2A, two lower bands with 395 bp and 117 bp in lane 2). To mimic the TNDM effect of *ZAC*, endogenous expression in normal rat islets would be a more meaningful overexpression target. For an approximate comparison of Tet-induced *ZAC* expression with endogenous *Lot1* expression in rat islets, we performed competitive RT-PCR on equal mixtures of RNA from rat islet and INS-R9-2 cells treated with various doses of Dox for 48 h. By this approach, *Lot1* expression in rat islets (Fig. 2B, the upper band with 512 bp in lane 4) was found approximately equal to that of *ZAC,* induced by 1000 ng/ml Dox (Fig. 2B, the two lower bands with 395 bp and 117 bp in lane 4). Therefore, *Lot1* expression is downregulated in our transformed beta-cell line but maximal Dox induced *ZAC* expression approximately equal to that in normal rat beta-cells.

Effect of overexpression of ZAC on cell proliferation

Overexpression of *ZAC* induces cell growth arrest and apoptosis in some cell lines [5] but, in our experiment, it did not have a noteworthy effect on glucose-stimulated INS-R9-2 cell proliferation (Fig. 3A, B). It also did not induce morphological changes of apoptosis and caspase 3 activities also did not change. In addition, *ZAC* did not affect the apoptosis induced by high glucose concentration, after culturing cells at 24 mM glucose for 3 or 5 days (Fig. 3C).

Effect of ZAC overexpression on insulin secretion

Glucose-stimulated insulin secretion (GSIS) is well preserved in the INS-1 cell line [20]. Graded overexpression of *ZAC* impaired insulin secretion as much as $41.6 \pm 1.1\%$ at the highest Dox concentration (Fig. 4A). K^+-induced insulin secretion was inhibited to an extent comparable to that seen with glucose (Fig. 4B), indicating an effect downstream of beta-cell depolarization.

Effect of ZAC overexpression on intracellular calcium levels ($[Ca^{2+}]_i$)

To test whether the inhibited insulin secretion is due to reduced $[Ca^{2+}]_i$ response, the step next to the cell depolarization, we examined $[Ca^{2+}]_i$ in response to the same stimuli. Un-induced INS-R9-2 cells showed a slightly reduced $[Ca^{2+}]_i$ increase compared to the parental line and a slight delay in onset of the response ($p < 0.01$, Fig. 5). Maximal *ZAC* over-expression did not impair the $[Ca^{2+}]_i$ response, either in terms of the number of cells responding (Fig. 5D) or in the magnitude of the response (Fig. 5F). In fact, treatment with Dox *increased* the $[Ca^{2+}]_i$ response to elevated glucose concentrations in both parental cells and INS-R9-2 cells by 20 and 60% respectively. Therefore, the effect on GSIS cannot be explained on the basis of a defect at the Ca^{2+} signalling level, suggesting an effect on downstream steps.

Effect of overexpression of ZAC on proinsulin biosynthesis

Although insulin mRNA was clearly increased in the presence of Dox (Fig. 6A), insulin content in cells did not substantially change with Dox, showing a tendency to increase at 48 h, but decreased ($12.2 \pm 5.5\%$) at 96 h with Dox.

We next examined the effect of Dox treatment on proinsulin biosynthesis. As expected, glucose (16.7 mM) increased total protein and proinsulin biosynthesis (Fig. 6B, C). Induced *ZAC* expression caused a decrease of proinsulin biosynthesis (Fig. 6B), but not the total protein biosynthesis (Fig. 6C). Normalization of the proinsulin biosynthesis to total protein biosynthesis showed that *ZAC* expression decreased proinsulin biosynthesis at high glucose (16.7 mM), but not at low (0.5 mM) glucose (Fig. 6D). Thus, overexpression of *ZAC* impaired glucose-stimulated proinsulin biosynthesis despite increasing insulin mRNA levels.

Glucose down-regulates Lot1/Zac1 expression in INS-1 cells and mouse islets

These findings on GSIS and insulin biosynthesis, suggest that *ZAC* may play an important role in glucose regulatory pathway. We examined *Lot1* expression in INS-1 cells at 5 and 11 mM glucose. Glucose decreased *Lot1* expression dramatically (Fig. 7A). In mouse islets, elevated glucose reduced *Zac1* expression although to a smaller effect than in INS-1 cells (Fig. 7B).

Discussion

We show here that induced increases in *ZAC* expression in INS-1 cells, to levels not substantially higher than physiologic (as judged at the mRNA level), reduces GSIS by almost half without significantly affecting apoptosis or proliferation, either basal or glucose-stimulated. In addition, we observed reduced insulin peptide synthesis in the face of increased insulin mRNA levels. This combination of results strongly suggests an action of ZAC on the efficiency with which insulin mRNA is translated, and suggests ZAC as a regulator of the corresponding translational machinery. One possible limitation of our experimental system is that INS1 cells have intrinsically very low ZAC expression. However, their GSIS closely parallels that of physiological islets and we have shown that is affected by additional expression of ZAC that differs little from physiological.

The effects of *ZAC* we have observed may explain at least one aspect of human TNDM, namely the mild insulin deficiency which is observed later in life. An explanation of the phenotype of total insulin deficiency in the neonate, associated with a transient near-total absence of insulin-staining cells in the one case that came to autopsy [21], requires additional effects on beta-cell development, proliferation or apoptosis. This could not be tested in INS-1, a transformed cell line.

Glucose triggers release of insulin stored in the secretory granules initially by serving as a substrate for phosphorylation by glucokinase. This leads to an increase in cytosolic ATP/ADP and the closing of ATP-sensitive K^+ channels (K_{ATP}) and cell doplarization. Ca^{2+} influx through voltage-gated Ca^{2+} channels, and granule exocytosis, then ensue [22]. Our results place the action of ZAC distal to Ca^{2+} channel function, i.e. at some aspects of granule exocytosis.

The nature of the stimulus-coupling signal pathways responsible for the observed effect of *ZAC* overexpression on proinsulin translation is similarly unclear. Although both processes require glucose metabolism, glucose-induced proinsulin biosynthesis is independent of extracellular Ca^{2+} [23]. ZAC, as a transcription factor and cofactor, may regulate insulin secretion and synthesis through a common mechanism, or through affecting specific events in each processes. Glucose can increase general protein biosynthesis by regulating the rate of initiation and translation elongation [24, 25] but proinsulin biosynthesis is specifically enhanced over and above the effect on general protein biosynthesis. Proinsulin biosynthesis depends on the interaction of the 5' UTR of its mRNA with a trans-acting factor [26] but it seems highly unlikely ZAC directly plays this role. An effect on translational regulators seems more likely.

Given our observation that *ZAC* expression acts as a negative regulator of GSIS and proinsulin biosynthesis, it is interesting to note that *ZAC* mRNA levels are down-regulated in response to high glucose. Since glucose modulates gene expression to maintain the glucose-responsive state [27, 28], it is possible that the down-regulation of *ZAC* expression may be an important event in glucose regulated insulin biosynthesis and secretion.

In conclusion, our results show that *ZAC* has complex effects on the beta-cell. The present data are consistent with, and complement, those we have previously obtained through over-expression of *ZAC* in a transgenic mouse [15]. Importantly, the present study has allowed us to explore the effects of this factor in an in vitro model for mature beta-cells without the potentially confounding effects of deletion at earlier developmental stages. Thus, we show in this simplified model that *ZAC* is a negative regulator of the effects of glucose on beta-cells, acting both on insulin biosynthesis and on a late event on insulin secretion, which lies downstream of cytosolic Ca^{2+} increases. We thus provide a possible explanation for the relapse of diabetes in TNDM cases. It will be necessary to further explore the precise molecular mechanisms that underlie *ZAC*'s effects, including the identification of *ZAC* target gene(s) and the signal transduction pathways involved. The possible role of *ZAC* in the beta-cell dysfunction of type 2 diabetes is worth pursuing and may suggest therapeutic interventions.

Acknowledgements

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Figure 1.

ZAC expression in beta-cell lines, mouse islets, and INS-R9-2 cells. (A) *ZAC* expression in different beta-cell lines and mouse islet by RT-PCR. INS-R9-2 cells were cultured in the presence or absence of doxycycline (Dox) for 48 h. *ZAC* expression was analyzed by RT-PCR (B) and real-time RT-PCR (C).

Figure 2.

Induced *ZAC* expression in INS-R9-2 cells in reference to *Lot1* expression endogenously and in normal rat islets. (A) Endogenous *Lot1* in INS-R9-2 cells compared to *ZAC* expression induced by Dox. For a competitive RT-PCR evaluation, products of RNA samples from INS-R9-2 cells, using primers that amplify both *Lot1* and *ZAC* and produce same length of PCR products (512 bp, in lane 1, 3, 5, 7), were digested by NdeI which cuts *ZAC* but not *Lot1.* PCR products of *ZAC* were cut into two fragments with 395 bp and 117 bp, those lower bands in lane 2, 4, 6, 8. (B) An approximate comparison of physiological level of *Lot1* expression in the normal rat islet vs. *ZAC* that induced by Dox in INS-R9-2 cells. We compared, by competitive RT-PCR, *ZAC* vs. *Lot1* level in an equal (by 18S) mixture of RNA from rat islets and INS-R9-2 cells stimulated by the indicated Dox concentrations. The two genes were distinguished by NdeI digestion. The lowest band in each lane is primer dimers. Ladder: 1 Kb Plus.

Figure 3.
Effects of *ZAC* overexpression on INS-R9-2 cell proliferation and apoptosis. ^{3}H Thymidine incorporation in cells cultured in standard medium (A), or in serum free medium for 24 h, then cultured at the indicated glucose concentration for another 24 h (B). (C) Cells were cultured in the indicated glucose concentration for the indicated days, and caspase 3 activity was tested. Data represent the mean ± SEM of three independent experiments done in triplicate. * P < 0.05, ** P < 0.01.

Figure 4.
Overexpression of *ZAC* impaired glucose-stimulated insulin secretion in INS-R9-2 cells.
Insulin secretion in response to 24 mM glucose (A) and 20 mM KCl (B). Data represent the
mean ± SEM of three or five independent experiments done in triplicate. *P < 0.05

Figure 5.
Overexpression of *ZAC* did not reduce glucose-induced intracellular Ca^{2+} increases. (A, B) Representative Ca^{2+} traces from single cells. (C, D) % of total live cells responding to glucose. (E, F) Area under the curve. Data are expressed as the mean of three separate experiments $\pm$ SEM. The total number of cells per condition was 67-121. * $P < 0.001$

Figure 6.

Overexpression of *ZAC* inhibited proinsulin biosynthesis. (A) INS-R9-2 cells were cultured with or without Dox for 48 h and 96 h, and *Ins2* mRNA was examined by real-time PCR. (B) INS-R9-2 cells were cultured with or without Dox for 96 h, cells were pulse labeled with ^{35}S methionine/cysteine for 30 min. Equal amounts of lysate according to total protein were subjected to immunoprecipitation by proinsulin antibody and analyzed by tricine SDS-PAGE. (C) Total protein biosynthesis was determined by TCA precipitation. (D) Specific proinsulin biosynthesis was obtained through normalizing proinsulin biosynthesis in (B) by the total protein synthesis in (C). (E) Specific proinsulin biosynthesis presented as the fold induction at high glucose relative to the level at low glucose. Data represent the mean ± SEM of three independent experiments done in triplicate. *P < 0.05 compared to in the absence of Dox.

Figure 7.
Glucose-regulated *Lot1/Zac1* expression in INS-1 cells and mouse islets. (A) INS-1 cells were cultured at 5 and 11 mM glucose for 24 h and 72 h. Real time RT-PCR show *Lot1* expression (n = 3). (B) Mouse islets were isolated and cultured at glucose concentration 3 and 11 mM for 24 h, real time RT-PCR show *Zac1* expression (n = 5). *P < 0.05 compared to the 5 mM or 3 mM glucose condition.