

Research Article

α -catenin isoforms are regulated by glucose and involved in regulating insulin secretion in rat clonal β -cell models

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The recent finding that β -catenin levels play an important rate-limiting role in processes regulating insulin secretion lead us to investigate whether its binding partner α -catenin also plays a role in this process. We find that levels of both α -E-catenin and α -N-catenin are rapidly up-regulated as levels of glucose are increased in rat clonal β -cell models INS-1E and INS-832/3. Lowering in levels of either α -catenin isoform using siRNA resulted in significant increases in glucose stimulated insulin secretion (GSIS) and this effect was attenuated when β -catenin levels were lowered indicating these proteins have opposing effects on insulin release. This effect of α -catenin knockdown on GSIS was not due to increases in insulin expression but was associated with increases in calcium influx into cells. Moreover, simultaneous depletion of α -E catenin and α -N catenin decreased the actin polymerisation to a similar degree as latrunculin treatment and inhibition of ARP 2/3 mediated actin branching with CK666 attenuated the α -catenin depletion effect on GSIS. This suggests α -catenin mediated actin remodelling may be involved in the regulation of insulin secretion. Together this indicates that α -catenin and β -catenin can play opposing roles in regulating insulin secretion, with some degree of functional redundancy in roles of α -E-catenin and α -N-catenin. The finding that, at least in β -cell models, the levels of each can be regulated in the longer term by glucose also provides a potential mechanism by which sustained changes in glucose levels might impact on the magnitude of GSIS.

Introduction

The proper control of glucose homeostasis relies on the appropriate levels of insulin being secreted from the β -cells in the pancreas so there has been an intense effort to understand the mechanisms by which glucose levels control insulin secretion [1,2]. Some parts of the process are understood. It is clear that the initial retention of insulin containing vesicles inside the cells and their release upon glucose stimulation involves regulation of the microtubules and the actin cytoskeletons [3,4]. The acute release of insulin upon glucose stimulation requires ATP production and subsequent closure of the ATP sensitive K^+ channel. The resulting membrane depolarisation leads to increased activity of voltage sensitive L-type calcium channels and the consequent influx of calcium allows t-snare/v-snare interactions that permits insulin granules to fuse with the plasma membrane [1,2].

β -cells are also a polarised cell type and this is important for proper GSIS [5–7]. They have membrane domains facing the blood vessels that interact with the extracellular matrix via focal adhesions and these are important for proper regulation of GSIS [8,9]. The lateral domains play an equally important role and appear to be where most of the glucose transporters and calcium channels are located [6,7]. It is clear that adherens junctions play an important role in forming interactions of lateral domains and there is a growing body of evidence that these adherens junctions also play an

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important role in the processes allowing the β -cells to release insulin in response to changes in extracellular glucose concentration [5,10–15]. These studies were initially restricted to showing a requirement for the cell surface cadherins but we have recently begun to investigate the role of the intracellular proteins that associate with cadherins and mediate their effects in the cell. The two most well studied components of these complexes are β -catenin, which binds directly to the cadherins, and α -catenin, which binds indirectly to the cadherins by binding first to β -catenin [16]. We have previously shown that loss of β -catenin results in attenuation of GSIS suggesting an important role for this protein in this process [17,18]. These effects appear to be independent of transcriptional effects of β -catenin but the full mechanisms remain to be elucidated, including the role of other proteins associated with β -catenin such as α -catenin.

One interesting finding of our previous work is that glucose exposure rapidly increases β -catenin levels in β -cells [17,18]. We have also recently shown that β -catenin levels increase rapidly in several regions of the hypothalamus after feeding which raises the possibility that such mechanisms may be important in regulating secretion of other hormones regulating energy metabolism [19]. This suggests that dynamic regulation of this protein could be playing a role in modulating the potential of β -cells to release insulin. The levels of β -catenin are regulated by ubiquitination and proteasomal degradation and the association of α -catenin with β -catenin is important in modulating this process [16].

Here we investigate the role of α -catenin in β -cell models INS-1E and INS-832/3. We find that levels of α -catenin isoforms in β -cell models rise following glucose exposure in a similar time frame to which β -catenin levels rise. However, we find loss of α -catenin has the opposite effect to loss of β -catenin and causes large increases in glucose stimulated release of insulin from β -cells. These increases are associated with parallel increases in voltage dependent calcium influxes into the β -cells lacking α -catenin and these are necessary for the increased insulin secretion. Moreover, α -catenin depletion in INS-1E cells decreases the actin polymerisation suggesting that α -catenin mediated regulation of GSIS may occur through modulation of actin cytoskeleton.

Experimental procedures

Cell culture

INS-1E cells were kindly provided by Professor C. B. Wollheim and INS-1 832/3 cells were kindly provided by Professor Christopher B. Newgard. INS-1E and INS-832/3 β -cells were maintained in RPMI 1640 medium containing 11.1 mM glucose, supplemented with 10% (v/v) FBS (fetal bovine serum), 100 units/ml penicillin, 100 μ g/ml streptomycin, 10 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate (all Gibco, Life Technologies), and 50 μ M 2-mercaptoethanol. Glucose treatment experiments were performed in confluent cells in six well culture plates and insulin secretion experiments were performed on >80% confluent cells in 12-well culture plates after serum-starvation in Krebs-Ringer Bicarbonate Hepes buffer (KRBH: 119 mM NaCl, 4.74 mM KCl, 1.19 mM MgSO_4 , 25 mM NaHCO_3 , 1.19 mM KH_2PO_4 , 2.54 mM CaCl_2 and 50 mM Hepes), pH 7.4, with 0.2% BSA (low fatty acid).

Cell transfection

All siRNAs and siRNA transfection reagents were purchased from Life Technologies and used according to manufacturer's instructions. All siRNA transfections were performed using reverse transfection method with either negative control siRNA (StealthTM RNAi siRNA Negative Control, Med GC) or siRNA specific for α -E catenin (RSS 358211, RSS 316764, RSS 316766), α -N catenin (RSS 311110, RSS 311111, RSS 355878) or β -catenin (RSS331357) at a final concentration of 30 nM using LipofectamineTM 2000 transfection reagent. 5×10^5 INS-1E cells were used in each well of 12 well plate and cells were plated in media lacking antibiotics. 24 h after transfection, medium was replaced with RMPI with antibiotics and experiments were performed 48 h after transfection.

Cell lysate preparation for western blot

After treatments, cells were rinsed with ice-cold 1xPBS and lysates were collected in buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerol phosphate, 1 mM vanadate, 100 mM NaF, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AESBF), 4 μ g/ml aprotinin, 0.4 μ g/ml pepstatin, 4 μ g/ml leupeptin, and 30 μ M N-[N-(N-Acetyl-L-leucyl)-L-leucyl]-L-norleucine (ALLN). Lysates were centrifuged at 16 100 \times g for 10 min, and supernatants were analysed by polyacrylamide gel electrophoresis for Western blotting.

Western blot analysis

Western blot analysis was done using antibodies against α -E catenin (1 : 1000; Cell Signalling Technologies, catalogue number # 240) and α -N catenin (1;1000; Cell Signalling Technologies, catalogue number 2163) and total β -catenin (1 : 2000; Symansis, catalogue number 3024C), α -tubulin (1 : 20 000; Sigma–Aldrich, catalogue number T6074) and β -actin (1 : 20 000; Sigma–Aldrich, catalogue number A1978). After overnight incubation at 4°C with primary antibodies, membranes were washed and incubated with respective secondary antibodies anti-mouse IgG HRP (1 : 20 000; Sigma–Aldrich), anti-rabbit IgG HRP (1 : 10 000 Santa Cruz biotechnology) or anti-sheep IgG HRP (1 : 20 000; Dako) for 1 h at room temperature and developed with Clarity™ Western ECL substrate (Bio-Rad Laboratories).

Measuring insulin concentrations

Forty-eight hours after siRNA transfection, cells were starved in KRBH buffer. After starvation, media were replaced with KRBH containing either 0.5 mM or 10 mM glucose for 2 h. To measure the amount of secreted insulin levels, aliquots of supernatants were collected, and insulin concentrations were measured using the AlphaLISA Insulin Assay kit (PerkinElmer) according to the manufacturer's instructions. To measure the total insulin content, cell lysates were collected, diluted accordingly and performed AlphaLISA insulin assay.

F actin G actin assay

INS-1E cells were plated in 12 well plate during siRNA transfection. Forty-eight hours after siRNA transfection, cells were washed with PBS and lysed with actin stabilisation buffer 50 mM NaCl, 5 mM MgCl₂, 50 mM PIPES, 5 mM EGTA, 5% (v/v) glycerol, 0.1% Nonidet P-40, pH 6.9, 0.1% Triton X-100, 0.1% Tween 20, 0.1%

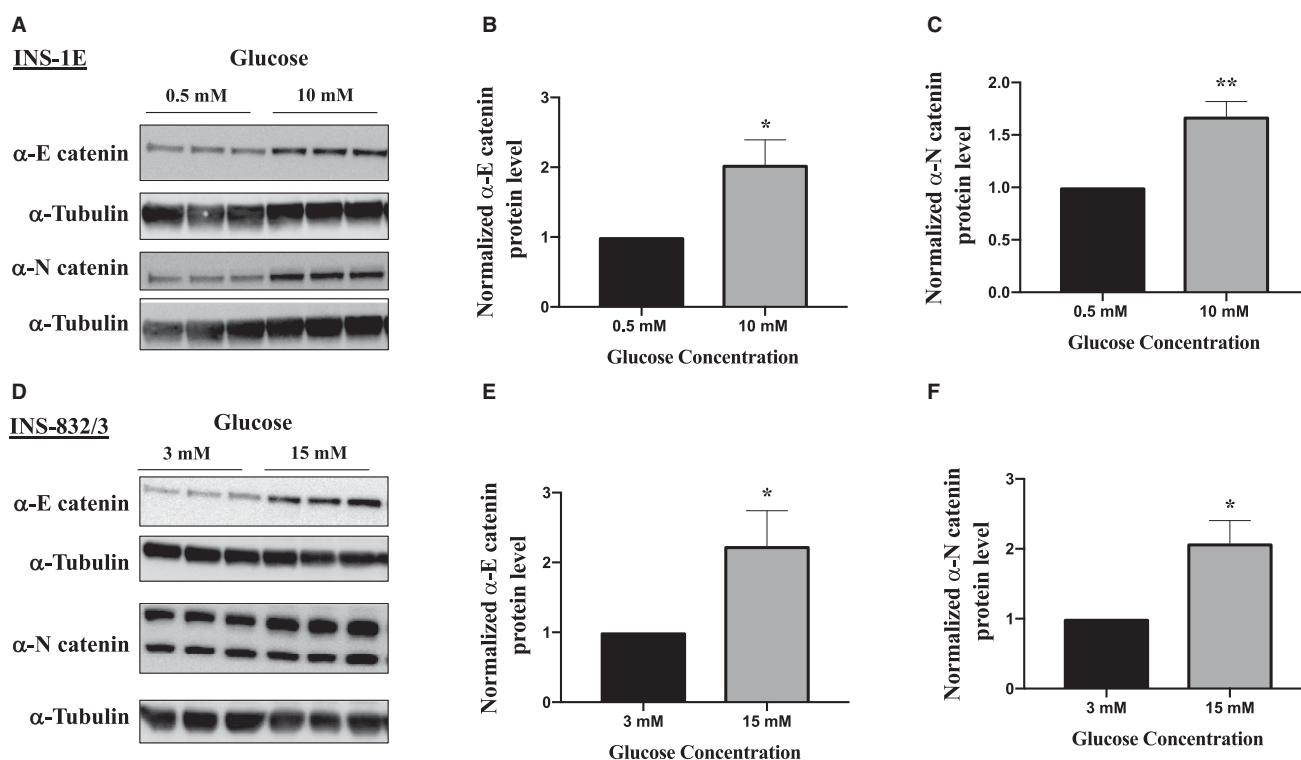


Figure 1. α -E catenin and α -N catenin protein levels increase with glucose stimulation.

After serum starvation in KRBH buffer, INS-1E cells (A) and INS 832/3 cells (D) were stimulated with 10 mM glucose or 15 mM, respectively for 3 h and lysates were subjected to western blot analysis using α -tubulin as a loading control. (B and C) Densitometry analysis of α -catenin protein levels in western blot images of INS-1E lysates and (E and F) INS-832/3 lysates. Results are mean \pm S.E.M of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ compared with control glucose condition as assessed by unpaired t -test (1B,C,E,F). Results are representative of least three independent experiments.

2-mercaptoethanol, 0.001% Antifoam A, 1 mM ATP, and protease inhibitors. Cells were collected by scraping and homogenised by passing through a 24-gauge needle five times. Cell lysates were incubated at 37°C for 10 min and centrifuged at 2000×g for 5 min to separate the cell debris. The G-actin was separated from F-actin by centrifuging at 10 000×g for 1 h at 37°C. F-actin pellet was resuspended in Milli-Q water containing 10 μM of cytochalasin D. Latrunculin-A was used as a negative control that reduces actin polymerisation and phalloidin was used as a positive control for actin polymerisation. F-actin and G-actin samples were run on 8% SDS PAGE gel and the expression of α-E catenin, α-N catenin and β-actin proteins were analysed.

Statistical analysis

Results are presented as means ± S.E.M. Statistical differences were determined using two-way ANOVA with Dunnett's or Tukey's post hoc test or unpaired t-tests and statistical significance is displayed as * $P < 0.05$ or ** $P < 0.01$. Statistical analyses were performed using statistical software package GraphPad Prism 6.0 (GraphPad Software Inc.).

Results

We find that increasing levels of glucose in INS-1E cells from 0.5 mM to 10 mM resulted in an increase in the levels of both α-E and α-N catenin in these cells which broadly parallels the increases we observe in β-catenin

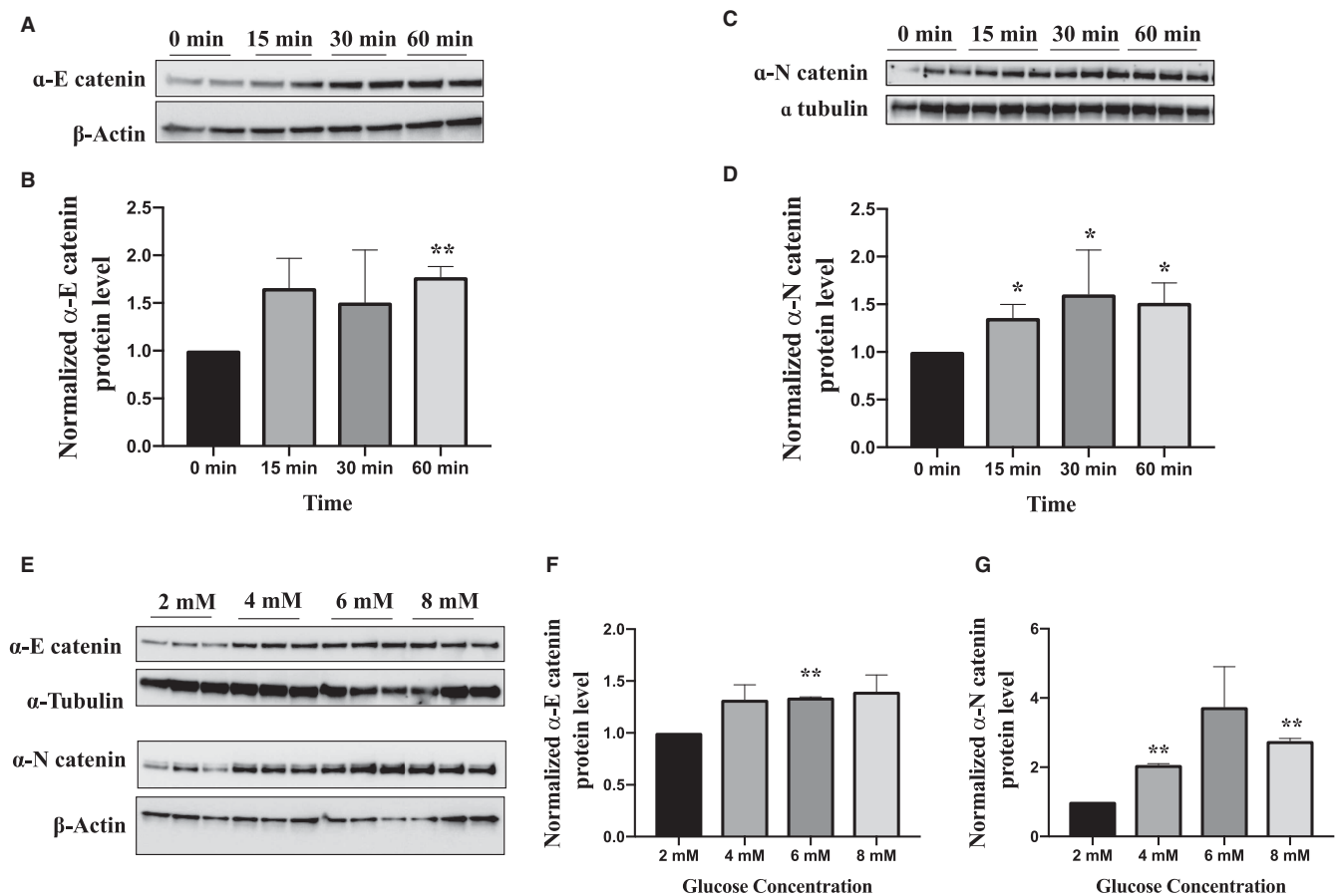


Figure 2. α-catenin responds to glucose at physiologically relevant concentrations and in a time dependent manner.

(A–D) Following serum starvation in KRBH buffer, INS-1E cells were stimulated with 10 mM glucose for the indicated time. Cell lysates were subjected for western blot analysis using α-E catenin, α-N catenin antibodies and β-actin and α-tubulin as loading controls. (E–G) After serum starvation, INS-1E cells were stimulated with increasing concentrations of glucose ranging from 2 mM–8 mM for 3 h and cell lysates were subjected for western blot analysis. Results are representative of at least three independent experiments. Results are mean ± S.E.M. of 2–4 independent experiments * $P < 0.05$ and ** $P < 0.01$ compared with control glucose condition as assessed by unpaired t-test (1B,D,F,G).

(Figure 1A–C). Similar results were seen in the INS-1 832/3 cells (Figure 1D–F). These increases occur in a time dependent manner (Figure 2A–D) and across a physiologically relevant range of glucose concentrations (Figure 2E–G) suggesting their potential involvement in insulin secretion process. Furthermore, we see an increase in α -N catenin levels when α -E catenin is depleted in these cells using siRNA but not an increase in α -E catenin when α -N catenin is depleted (Figure 4A) indicating that α -N catenin might compensate for the loss of α -E catenin in this system.

We independently investigated the roles of both of the isoforms of α -catenin in insulin secretion. Depletion of each isoform alone surprisingly resulted in an increase in GSIS in INS-1E (Figure 3A–D). Similar results were found in INS-1 832/3 cells (data not shown). Simultaneous depletion of both α -catenin isoforms had an additive increase on GSIS (Figure 4A,B). This effect of α -catenin depletion was the opposite of what we find when we deplete β -catenin, therefore we investigated the effect of simultaneous knockdown of both α - and β -catenin (Figure 4C,D). In these experiments we find that α -catenin induced increase in GSIS was almost totally attenuated when β -catenin was also knocked down. To investigate the mechanism by which α -catenin might be regulating insulin secretion we investigated the possibility that α -catenin was increasing levels of insulin overall in the β -cells but this was not the case (Figure 5A). We next investigated whether loss of α -catenin affected calcium influx into the cells. We used FLIPR to investigate the effects on voltage sensitive calcium influx into the β -cells initiated by K^+ induced depolarisation. We find that KCl mediated increases in insulin release were increased in α -catenin depleted cells (Figure 5B) and that this is associated with a large increase in calcium influx into the cells (Figure 5C). Consistent with this influx in calcium being linked to the increase in insulin secretion we find that the calcium channel blocker nifedipene largely attenuated the increases in insulin secretion in the α -catenin depleted cells at a concentration of drug at which it is selective for L-type channels (Figure 5D).

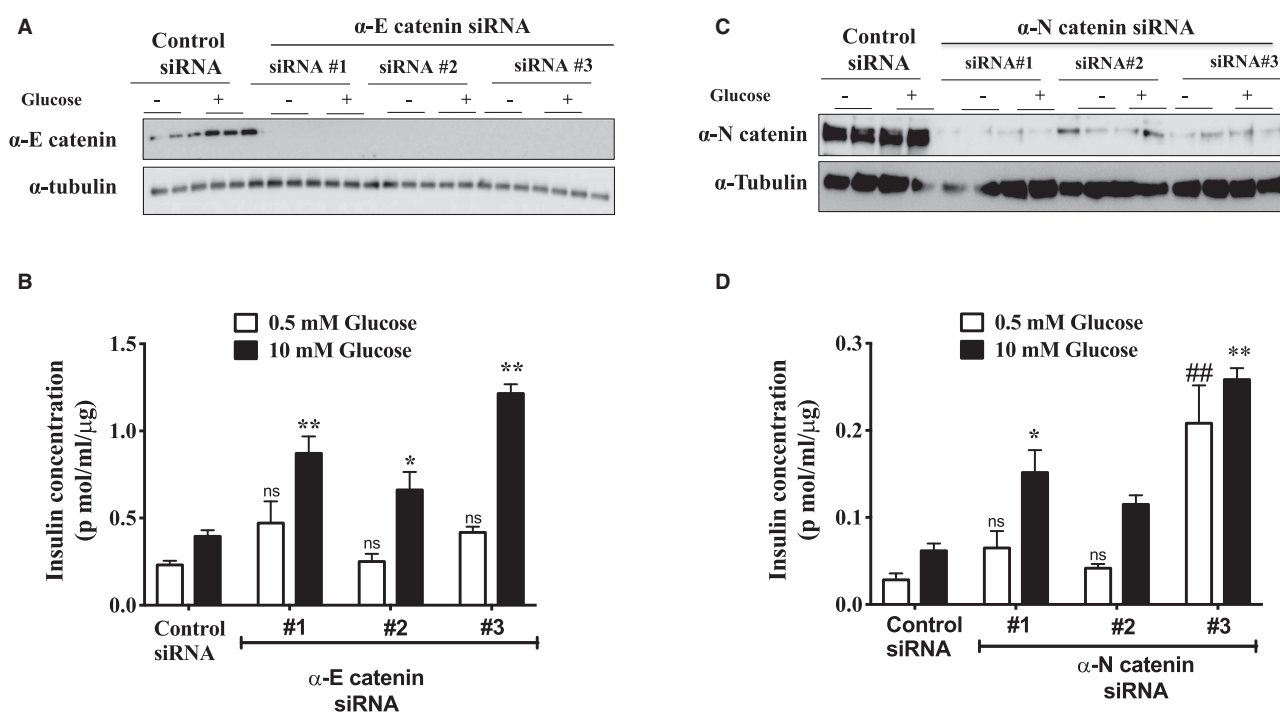


Figure 3. α -catenin involve in the negative feedback mechanism during glucose stimulated insulin secretion.

INS-1E cells were transfected with either control siRNA or siRNA specific for α -E catenin or α -N catenin. Forty-eight hours after siRNA transfection, cells were serum starved for 2 h and stimulated with either 0.5 mM Glucose or 10 mM glucose for 2 h. Insulin concentrations were measured by performing alphaLIZA assays and were normalised to total protein content. (A) Immunoblot analysis of α -E catenin levels in INS-1E lysates after siRNA transfection and (B) insulin concentrations in supernatants of negative control or α -E catenin siRNA transfected cells. (C) Immunoblot analysis of α -N catenin protein level in INS-1E lysates after siRNA transfection and (D) insulin concentrations in supernatants of negative control or α -N catenin siRNA transfected cells. Results are mean \pm S.E.M. * $P < 0.05$ and ** $P < 0.01$ compared with control glucose stimulated condition as assessed by two-way ANOVA. Similar results were obtained in at least three independent experiments.

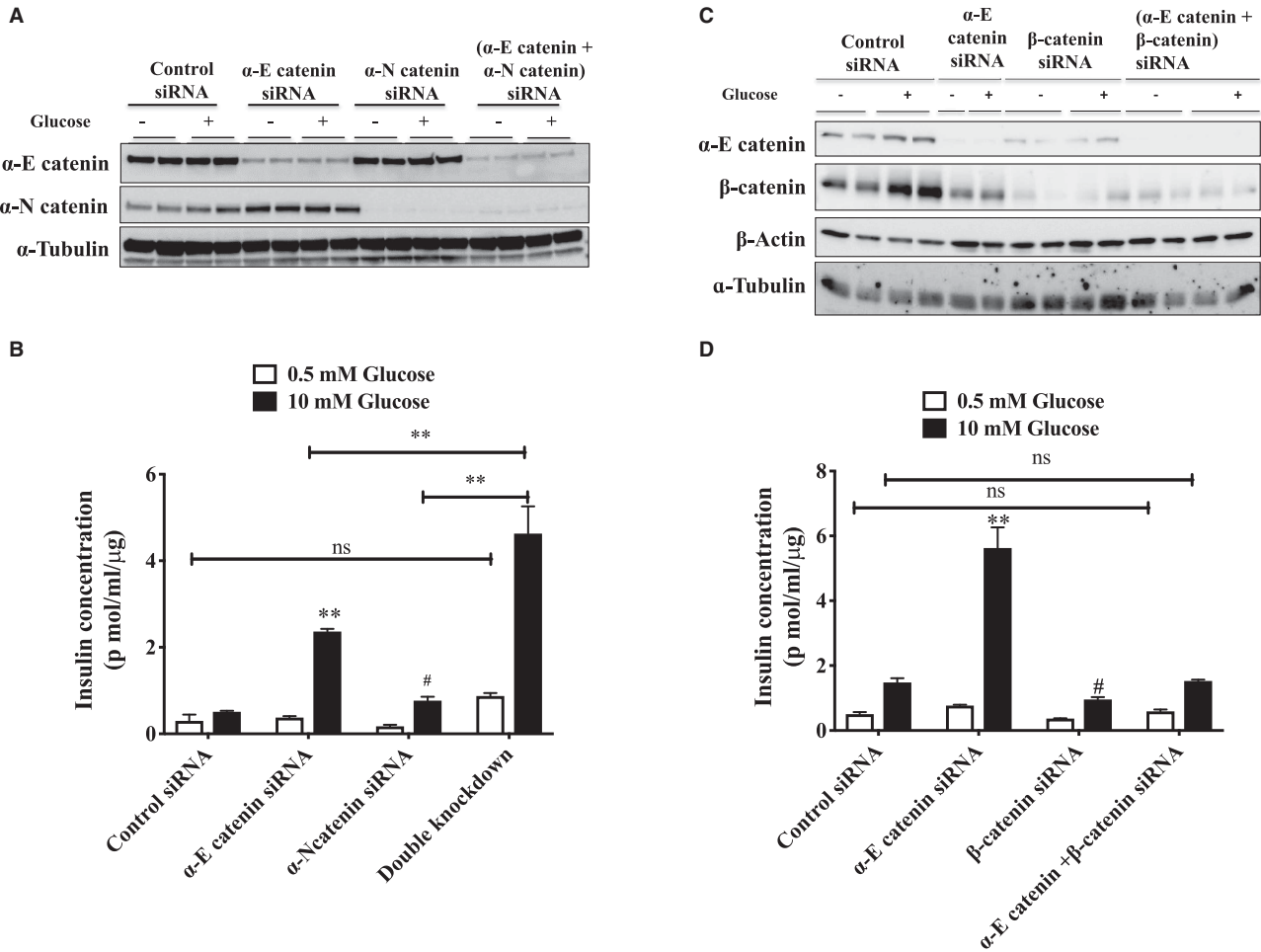


Figure 4. α -E catenin and α -N catenin is functionally redundant in β -cells.

INS-1E cells were transfected with negative control siRNA, α -E catenin siRNA, α -N-catenin siRNA or a combination of both α -E catenin and α -N-catenin siRNA. (A) Immunoblot analysis of INS-1E lysates after siRNA transfection using α -E catenin antibody, α -N catenin antibody and α -Tubulin. (B) Insulin concentrations in supernatants of siRNA transfected cells. β -catenin depletion attenuates α -E catenin knockdown effect on glucose stimulated insulin secretion. INS-1E cells were transfected with negative control siRNA, α -E catenin siRNA, β -catenin siRNA or a combination of both α -E catenin and β -catenin siRNA. (C) Immunoblot analysis of INS-1E lysates after siRNA transfection. (D) Insulin concentrations in supernatants of siRNA transfected cells. Results are mean \pm S.E.M. * $P < 0.05$ and ** $P < 0.01$ compared with control siRNA. glucose stimulated condition as assessed by two-way ANOVA. # $P < 0.05$ compared with control siRNA glucose stimulated condition as assessed by t -test. Similar results were obtained in at least three independent experiments.

As α -catenin is a well-known actin cytoskeleton regulator, we investigated the effect of α -catenin depletion on actin polymerisation. When we simultaneously knockdown α -E catenin with α -N catenin we find that the ratio between filamentous (F)-actin and monomeric (G)-actin is significantly reduced (Figure 6A,B), similar to the effect of latrunculin which is also known to potentiate the GSIS [20]. Furthermore when we inhibit actin branching with ARP 2/3 inhibitor CK666, α -catenin knockdown effect on GSIS is attenuated indicating the α -catenin mediated regulation of insulin secretion potentially involves the regulation of branching of the actin cytoskeleton (Figure 6C,D).

Discussion

Adherens junctions play an important role in the regulation of some vesicle trafficking processes, including GSIS [5,18,21,22]. β -catenin mediates some effects of adherens junctions [23] and we have previously shown its is required for the proper translocation of GLUT4 containing vesicles in adipocytes [21] and of insulin

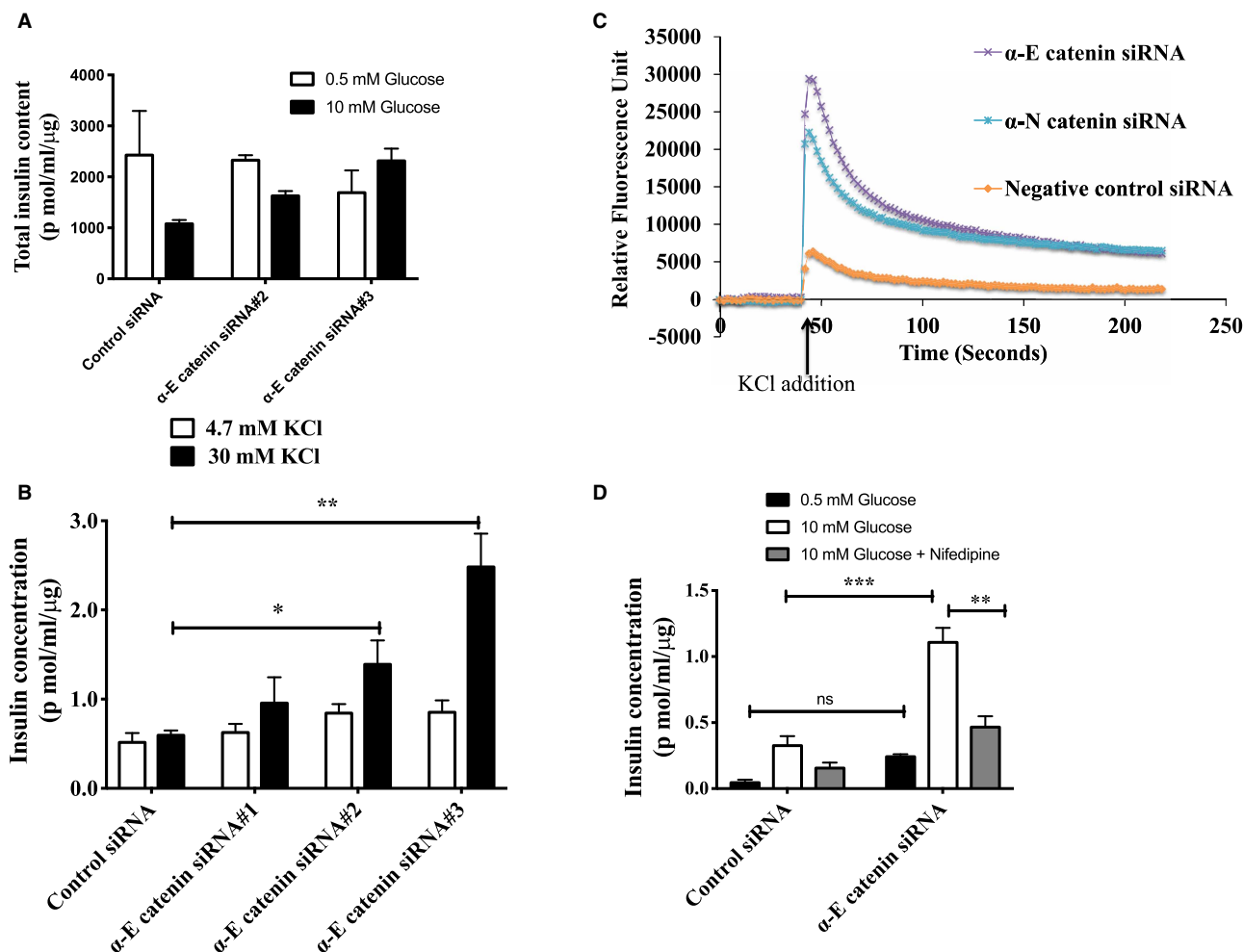


Figure 5. α -E catenin depletion affects Calcium influx to β -cells.

(A) Forty-eight hours after siRNA transfection total insulin contents in the cell lysates were measured by alphaLISA and normalised to total protein content. Similar results were obtained in at least two independent experiments. (B) INS-1E cells were transfected with either control siRNA or siRNA specific for α -E catenin. Forty-eight hours after siRNA transfection, cells were serum starved for 2 h and stimulated with either 4.7 mM KCl or 30 mM KCl for 2 h. Insulin concentrations were measured by performing alphaLISA assays and were normalised to total protein content. Results are mean \pm S.E.M. * $P < 0.05$ and ** $P < 0.01$ compared with control/low glucose condition as assessed by two-way ANOVA. Similar results were obtained in at least three independent experiments. (C) α -catenin depletion increases the K^+ stimulated Calcium flux into β -cells. INS-1E cells were transfected either with negative control siRNA or siRNA specific for either α -E catenin or α -N catenin. Forty-eight hours after siRNA transfection cells were incubated with calcium binding dye for 1 h at 37°C and readings were taken for 225 s at 2 s intervals. At 40 s, 10 μ l of KCl was added to each well, such that the final KCl concentration is 30 mM. Relative Fluorescence units (RFU) were calculated by subtracting each fluorescence signal from initial fluorescence signal in different siRNA conditions. Similar results were obtained in at least three independent experiments. (D) Nifedipine treatment attenuates the α -E catenin knockdown effect on K^+ stimulated insulin secretion. Forty-eight hours after siRNA transfection cells were stimulated with 10 mM glucose in the presence or absence of Calcium channel inhibitor-Nifedipine. Insulin concentrations were measured by performing alphaLISA assays and were normalised to total protein content. Results are mean \pm S.E.M. * $P < 0.05$ and ** $P < 0.01$ compared with control/low glucose condition as assessed by two-way ANOVA.

containing vesicles in β -cells [18]. The current study further explores the mechanisms involved by examining the role of the β -catenin binding partner α -catenin in GSIS. These studies show α -catenin is involved in negative feedback regulation of GSIS. At least in these these rat clonal model β -cell models the most likely interpretation of our data is that both α -E-catenin and α -N-catenin isoforms play functionally redundant roles in this process. We find that lowering α -catenin levels has a similar effect on insulin secretion and actin

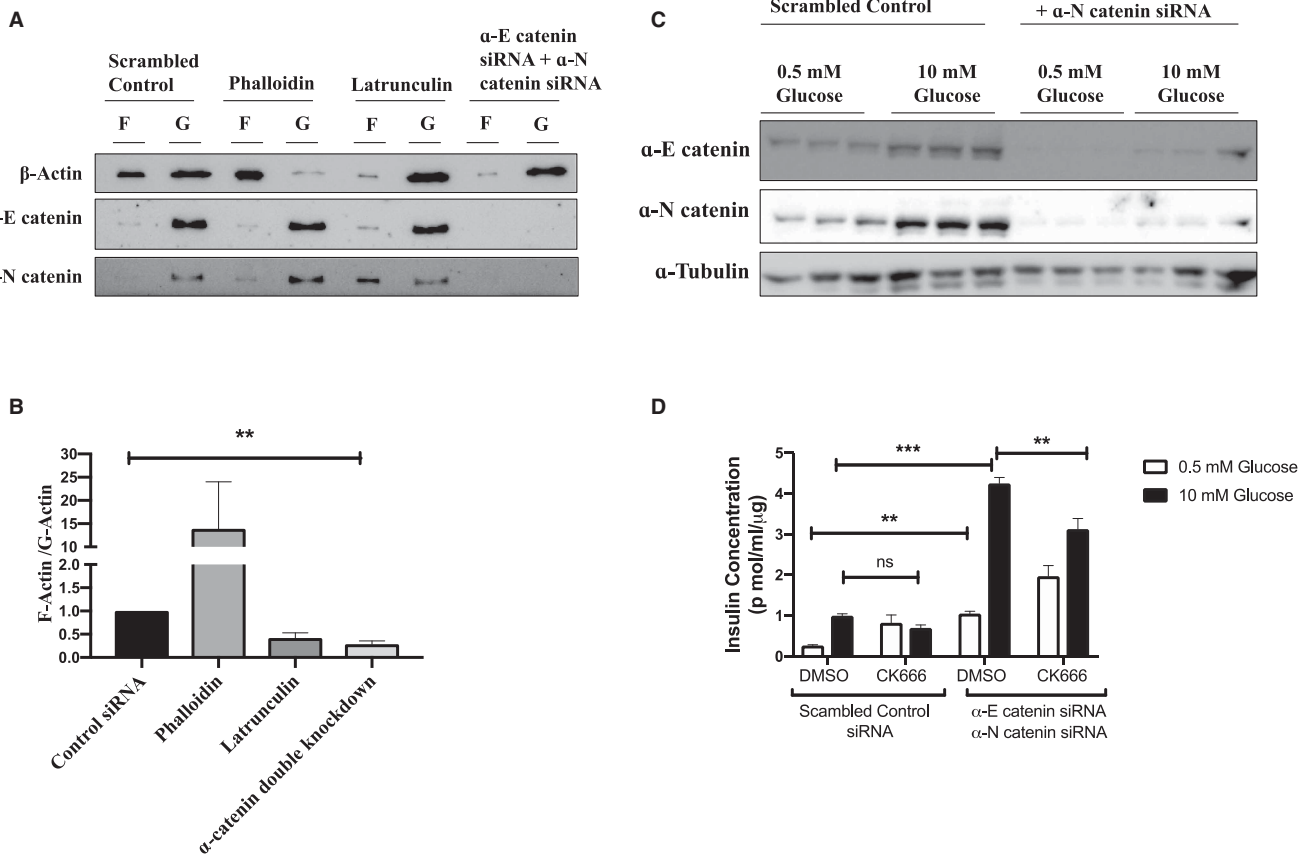


Figure 6. α -catenin depletion affects actin polymerisation in INS-1E cells.

INS-1E cells were transfected with either control siRNA or siRNA specific for α -E catenin and α -N catenin siRNA. Phalloidin was used as a positive control for actin polymerisation while latrunculin was used as a negative control. (A) F-actin was separated from G-actin by ultracentrifugation and expression of F-actin and G-actin were analysed by performing western blot analysis using β -actin antibody. (B) F-actin and G-actin ratio was analysed by densitometry. Results are mean \pm S.E.M of four independent experiments. * $P < 0.05$ and ** $P < 0.01$ compared with control siRNA as assessed by unpaired t -test. INS-1E cells were transfected with either control siRNA or siRNA specific for α -E catenin and α -N catenin. Forty-eight hours after siRNA transfection cells were serum starved and stimulated with 10 mM Glucose in the presence of either DMSO or CK666. (C) Lysates were used for western blot analysis to confirm the knockdown. (D) AlphaLIZA assays were performed to measure the secreted insulin concentrations. Results are mean \pm S.E.M. * $P < 0.05$ and ** $P < 0.01$ compared with control/low glucose condition as assessed by two-way ANOVA.

polymerisation to that classically seen with latrunculin suggesting the α -catenin is important in maintaining appropriate cytoskeletal architecture to achieve proper regulation of insulin secretion.

Our evidence indicates that one role of the α -catenin is to regulate the processes regulating calcium levels in the β -cells following membrane depolarisation. Consistent with this it has previously been shown that dispersed β -cells have spontaneous fluctuations in intracellular calcium levels while stable calcium levels are seen in confluent β -cells [24]. The confluent β -cells have adherens junctions which provide a pool of α -catenin localised near the plasma membrane [25] and this could be involved in restraining the calcium fluxes. We have previously shown that lowering β -catenin levels attenuates glucose and KCl mediated release of insulin [17,18] but we did not find any effect of lowering β -catenin on calcium influx.

We find that reductions in β -catenin strongly attenuate the increases in GSIS seen when α -catenin levels are reduced indicating that the requirement for β -catenin in insulin secretion dominates over the effect of α -catenin. This suggests it is possible that the pool of β -catenin/ α -catenin heterodimers act separately from α -catenin homodimers. The dimeric form of α -catenin is known to play a key role in regulating the actin cytoskeleton, a process known to be important in the control of insulin secretory vesicles [3,4]. Alpha-catenin dimers control the switch of actin from branch chains to linear cable structures by competing with for binding

with the ARP 2/3 protein [25]. Consistent with this it has previously been shown that knockdown of N-WASP, which is an activator of ARP 2/3, suppresses GSIS suggesting that activation of ARP 2/3 is important for proper GSIS [26]. In our system where we lower α -catenin levels there will be less inhibitory effect on ARP 2/3 leading to more branched actin filaments and less linear actin cables, which can potentially give easy access to insulin granules to fuse with the plasma membrane. In accordance with this hypothesis, we found that when we inhibit actin branching with ARP 2/3 inhibitor CK666 in an α -catenin depleted system the α -catenin knock-down effect on insulin secretion is reduced. Overall these findings suggest that α -catenin mediated regulation of GSIS may occur through modulation of actin dynamics though the details by which α -catenin regulates GSIS remain to be resolved in future studies.

Our findings here that levels of α -catenin proteins are important in regulating insulin secretion means that factors controlling levels of these proteins have the potential to impact on the ability of β -cells to release insulin. Our finding that α -catenin levels change rapidly in response to changes in glucose in β -cell models thus suggests a mechanism by which high glucose levels might feed back to control levels of insulin secretion. This would potentially be important in fine-tuning insulin secretion in normal states but it would also be of interest to understand how changes in the levels of these proteins might be involved in causing the changes in β -cell function brought about by long term exposure to insulin resistant and diabetic states. Very little information is available on this although a redistribution of α -catenin away from adherens junctions has been observed in islets of mice fed a high fat diet [27].

Overall these studies show that α -catenin and β -catenin play opposing roles in regulating GSIS. The α -catenin is able to inhibit both calcium fluxes and insulin secretion while the β -catenin plays a separate role that is required for GSIS with the relative levels of each thus having potential to influence the overall ability of β -cells to secrete insulin after membrane depolarisation. However, since these studies are confined to rat clonal β -cell models, further studies will be required to understand whether these mechanisms play a role in the regulation of insulin secretion in functional islets where the proper cellular architecture will be present.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

Waruni Dissanayake, Brie Sorrenson, Kate Lee, and Peter Shepherd contributed to the experimental design. Waruni Dissanayake, Brie Sorrenson, Kate Lee, Sandra Barre performed the experiments. Waruni Dissanayake, Peter Shepherd analysed and interpreted the data. Waruni Dissanayake and Peter Shepherd wrote the paper.

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Abbreviations

FBS, fetal bovine serum; GSIS, glucose stimulated insulin secretion.

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