Hyperglycemia downregulates Connexin36 in pancreatic islets via the upregulation of ICER-1/ICER-1 γ

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Abstract

Channels formed by the gap junction protein Connexin36 (CX36) contribute to the proper control of insulin secretion. We previously demonstrated that chronic exposure to glucose decreases Cx36 levels in insulin-secreting cells *in vitro*. Here, we investigated whether hyperglycemia also regulates Cx36 *in vivo*. Using a model of continuous glucose infusion in adult rats, we showed that prolonged (24–48 h) hyperglycemia reduced the Cx36 gene *Gjd2* mRNA levels in pancreatic islets. Accordingly, prolonged exposure to high glucose concentrations also reduced the expression and function of Cx36 in the rat insulin-producing INS-1E cell line. The glucose effect was blocked after inhibition of the cAMP/PKA pathway and was associated with an overexpression of the inducible cAMP early repressor ICER-1/ICER-1 γ , which binds to a functional cAMP-response element in the promoter of the Cx36 gene *Gjd2*. The involvement of this repressor was further demonstrated using an antisense strategy of ICER-1 inhibition, which prevented glucose-induced downregulation of Cx36. The data indicate that chronic exposure to glucose alters the *in vivo* expression of Cx36 by the insulin-producing β -cells through ICER-1/ICER-1 γ overexpression. This mechanism may contribute to the reduced glucose sensitivity and altered insulin secretion, which contribute to the pathophysiology of diabetes.

Key Words

- ▶ gap junctions
- ▶ connexin36
- ▶ glucose
- ▶ ICER-1

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Introduction

Most cells communicate with each other by gap junctions, which provide a direct pathway for the intercellular diffusion of small cytosolic molecules (Bosco *et al.* 2011). Others and we have demonstrated that Connexin36 (Cx36) is the sole connexin expressed in the gap junctions of the insulin-producing β -cells (Theis *et al.* 2004, Serre-Beinier *et al.* 2009). The cell-to-cell communication mediated by Cx36 channels

is essential for the proper regulation of storage (Serre-Beinier *et al.* 2009) and release of insulin (Hamelin *et al.* 2009, Head *et al.* 2012) and contributes to the resistance of β -cells to aggressive conditions (Klee *et al.* 2011, Meda 2012, Haefliger *et al.* 2013). These results suggest that alterations of the endogenous levels of Cx36, e.g. after exposure to a diabetogenic environment, could have major effects on

β -cell function, thus contributing to the onset and progression of the disease (Hamelin *et al.* 2009, Bosco *et al.* 2011).

The prevalence of type 2 diabetes (T2D) is increasing at an alarming rate due to the combination of aging, urbanization, increasing prevalence of obesity, and physical inactivity (Wild *et al.* 2004). Chronic hyperglycemia and hyperlipidemia severely impact on β -cell function and survival, contributing to the progressive β -cell failure, which is central to the development of T2D and metabolic syndrome (Robertson 2009, Poitout *et al.* 2010). Still, the molecular and cellular mechanisms leading to the β -cell failure remain to be fully unraveled. The experimental evidence summarized above supports a likely role of Cx36. Prolonged *in vitro* exposure to high concentrations of glucose inhibits Cx36 expression in insulin-secreting cells (Allagnat *et al.* 2005), and Cx36 levels are decreased in hypercholesterolemic *Apoe*^{-/-} mice (Haefliger *et al.* 2013) or mice consuming a high-fat diet (Allagnat *et al.* 2008). Here, we studied whether hyperglycemia also resulted in Cx36 downregulation *in vivo*, using a model of continuous glucose infusion in adult rats (Bedoya & Jeanrenaud 1991a,b). We report that long-term glucose oversupply leads to a progressive downregulation of the islet Cx36 *in vivo*. We have recently shown that the Cx36 gene *Gjd2* is a target of the inducible cAMP early repressor ICER-1/ICER-1 γ (Allagnat *et al.* 2008), which is induced by glucose (Abderrahmani *et al.* 2006). Therefore, we further investigated whether ICER-1/ICER-1 γ was implicated in the glucose-induced downregulation of Cx36 in the insulin-producing INS-1E cell line. We report that the expression levels of ICER-1/ICER-1 γ are inversely related to those of Cx36 and that the repressor directly binds to the promoter of *Gjd2* to mediate the downregulation of Cx36 expression during a prolonged exposure to high glucose concentrations.

Materials and methods

Animal experiments

All experiments were conducted as per the rules and under the authorization of the Veterinary Office of the State of Geneva. Three–four-month-old male Sprague–Dawley rats (300–450 g) were anesthetized by inhalation of 3% isoflurane in air and equipped with a right femoral vein catheter as described previously (Bedoya & Jeanrenaud 1991a,b). After a week of recovery, during which the implanted catheter was infused with 0.9% NaCl containing 5 IU/ml heparin and 2000 IU/ml penicillin, the experimental infusion was started by a bolus injection,

via the implanted catheter, of 350 mg/kg D-glucose (given as a 40% w/v solution in 0.9% NaCl) and continued up to the end of the experiment by connecting the catheter to an infusion pump that delivered 180 mg/kg per min clinical-grade, pyrogen-free D-glucose (given as a 20% w/v solution) for 6, 12, 24, or 48 h. Controls received similar volumes of 0.9% NaCl. Four to seven rats were prepared per time point. Before starting the infusion, and periodically throughout it, blood samples were taken from the tail of each rat for the measurement of plasma glucose and insulin concentrations. Plasma concentrations of glucose were measured using the Ascencia DEX² blood glucose meter Bayer (Schweiz) AG, Zürich, Switzerland. Plasma insulin levels were determined using an ultra-sensitive rat insulin ELISA (Mercodia, Uppsala, Sweden). At the end of each infusion, rats were anesthetized by isoflurane ventilation, and their pancreas rapidly inflated through the main pancreatic duct, with a collagenase solution (Ravier *et al.* 2005, Klee *et al.* 2011). Islets of Langerhans were isolated, purified on histopaque gradients, and individually handpicked to minimize contamination by exocrine pancreatic remnants (Ravier *et al.* 2005, Klee *et al.* 2011).

Cell experiments

The rat insulinoma cell line INS-1E (kindly provided by Dr Pierre Maechler, CMU, University of Geneva) was maintained in the complete RPMI 1640 medium, containing 11 mmol/l glucose, as described previously (Merglen *et al.* 2004). Cells were kept at 37 °C in a humidified incubator under 5% CO₂ (v/v). Cultures were exposed for various periods of time to the same culture medium, containing glucose at concentrations varying from 5 to 25 mmol/l, as indicated. Some of these experiments were carried out in the presence of 5 μ M of the PKA inhibitor H89 (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland).

RNA isolation and quantitative RT-PCR (Lightcycler®)

mRNA of the isolated islets and INS-1E cells was isolated using nucleospin RNA II columns (MACHEREY-NAGEL AG, Oensingen, Switzerland) and the Tripure Isolation Reagent (Roche Diagnostics SA, Rotkreuz, Switzerland) respectively. Transcripts (1 μ g) were reverse-transcribed using the ImProm-2 Reverse transcription System (Promega AG, Dübendorf, Switzerland). Quantitative PCR was performed using the SYBR Premix ExTaq (Takara Bio Europe/SAS, Saint-Germain-en-Laye, France) in a

Lightcycler Instrument (Roche Diagnostics). cDNAs were amplified using the following primers: rat *Cx36 Gjd2*: 5'-ATACAGGTGTGAATGAGGGAGGATG-3' (sense) and 5'-TGGAGGGTGTACAGATGAAAGAGG-3' (antisense); rat ribosomal protein *Rpl27*: 5'-GATCCAAGATCAAGTCC TTTGTG-3' (sense) and 5'-CTGGGTCTCTGAACACATCC T-3' (antisense); *Ins2*: 5'-TGGCTTCTTCTACACACC-3' (sense) and 5'-TCTAGTTGCAGTAGTTCT-3' (antisense); *Icer1*: 5'-CTGGGT CTCTGAACACATCCT-3' (sense) and 5'-C ACCTGTGGCAAAGCAGTA-3' (antisense); *Rph3al*: 5'-GGC ACACTCTCTGGAGGAAG-3' (sense) and 5'-GGGGAGGGGC AATAAAT-AAA-3' (antisense); *Rab3a*: 5'-GTCAGCAC TGTGGGCATAGA-3' (sense) and 5'-TGCACTGCATTGAAG-GACTC-3' (antisense); and *Gapdh*: 5'-GACTCCACGACATAC TCAGC-3' (sense) and 5'-GTCGGTGTGAACGGATTGG-3' (antisense). All data were normalized to the housekeeping gene *Rpl27*, whose expression was constant in these experiments.

Western blotting

INS-1E cells were solubilized by sonication in SDS buffer (62.5 mmol/l Tris-EDTA, pH 6.8; SDS 5% v/v). Protein content was measured using a detergent-compatible BCA protein assay kit (Pierce, Thermo Fisher Scientific, Lausanne, Switzerland). Western blotting was carried out as described previously (Allagnat *et al.* 2005). Membranes were saturated for 1 h in TBS containing 5% w/v milk and 0.05% v/v Tween-20, prior to an overnight hybridization at 4 °C with either rabbit polyclonal antibodies against *Cx36* (Le Gurun *et al.* 2003, Martin *et al.* 2003), diluted 1:200, or monoclonal anti- α -tubulin antibodies (Fluka Chemie, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland, diluted 1:2000). CREM-1/ICER-1/ICER-1 γ western blotting was performed on nuclear extracts using the rabbit polyclonal anti-CREM-1 sc440 (Santa Cruz Biotechnology, Inc. Heidelberg, Germany, 1:2000) as described previously (Allagnat *et al.* 2008, Favre *et al.* 2011, Haeffliger *et al.* 2013). After incubation at room temperature (1 h) with a convenient secondary antibody conjugated to HRP (Fluka Chemie, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland, diluted 1:20 000), membranes were revealed by enhanced chemiluminescence (ECL, Amersham Biosciences, GE Healthcare Europe GmbH, Glattbrugg, Switzerland). Densitometric analyses of immunolabeled proteins (western blots films) were performed using the Image Quant Software (Molecular Dynamics, Amersham Biosciences, GE Healthcare Europe GmbH, Glattbrugg, Switzerland).

Transient transfection and luciferase assays

INS-1E cells plated in 24-well dishes were transiently transfected using Lipofectamine 2000 Reagent (Invitrogen) and a DNA:lipid ratio of 1:1, according to the manufacturer's instructions. All experiments involved the simultaneous transfection of a plasmid containing the mouse luciferase gene under the control of a 1 kb fragment of the human *GJD2* promoter, the internal control plasmid pRL-SV40 (Promega), and an empty vector (pCDNA3), a pCDNA3 allowing for the constitutive expression of ICER-1 (Molina *et al.* 1993), or a pCDNA3 coding for a ICER-1/ICER-1 γ antisense construct (Abderrahmani *et al.* 2006). Twenty-four hours after transfection, the cells were incubated at various glucose concentrations for another 24 h. Cells were lysed in 100 μ l ice-cold Passive Lysis Buffer for 15 min, and the *firefly* and *Renilla* luciferase activities were immediately measured using 50 μ l lysate and the Dual-Luciferase Reporter Assay System (Promega). The *Firefly* activity was normalized to the *Renilla* activity.

Chromatin immunoprecipitation assay

INS-1E cells (10^6 cells) were cross-linked with 1% formaldehyde at room temperature for 10 min. The cross-linking reaction was stopped by adding glycine to a final concentration of 125 nmol/l. Cells were collected and resuspended in the SDS lysis buffer (1% v/v SDS, 10 mmol/l EDTA, and 50 mmol/l Tris/HCl, pH 8.1). Cell pellets were lysed and sonicated to obtain the desired chromatin length (~ 500 bp). Protein concentration of the cell supernatants was determined by the BCA protein assay (Pierce, Thermo Fisher Scientific, Lausanne, Switzerland), and the samples were diluted to 1 mg/ml in the chromatin immunoprecipitation (ChIP) dilution buffer (1% v/v Triton X-100, 2 mmol/l EDTA, 150 mmol/l NaCl, 20 mmol/l Tris-HCl, pH 8.1, and protease inhibitors). Samples were precleared by incubation with blocked protein A Sepharose (Amersham Biosciences) for at least 1 h at 4 °C. The precleared chromatin lysates were immunoprecipitated overnight at 4 °C with polyclonal rabbit antibodies to CREM-1 (Santa Cruz Biotechnology), c-Myc (9E10, Santa Cruz Biotechnology), or human REST (Martin *et al.* 2003). The DNA-protein-antibody complexes were collected by the addition of protein A Sepharose for 2 h at 4 °C. Then, the DNA-protein complexes were washed twice in a low-salt wash buffer (0.1% v/v SDS, 1% v/v Triton X-100, 2 mmol/l EDTA, 20 mmol/l Tris-HCl, and 150 mmol/l NaCl) and once in a

high-salt final wash buffer (0.1% v/v SDS, 1% v/v Triton X-100, 2 mmol/l EDTA, 20 mmol/l Tris-HCl, and 500 mmol/l NaCl). The complexes were eluted from the sepharose beads by incubation at room temperature for 15 min in 1% v/v SDS and 100 mmol/l NaHCO₃. Eluates were heated for at least 4 h at 65 °C to reverse the formaldehyde cross-linking and precipitated overnight at –20 °C. The pellets were diluted in TE, RNase A- and Proteinase K-treated, and purified using the PCR product cleanup kit (Roche Diagnostics) according to the manufacturer's instructions. DNA was submitted to standard PCR amplification or lightcycler (one cycle at 95 °C for 3 min, followed by 32 cycles at 94 °C for 25 s, 58 °C for 25 s, 72 °C for 25 s, and finally one cycle at 72 °C for 3 min), using primers 5'-CACCAGCGTGTCTGTTCT-3' (sense) and 5'-ATCTTGCGGTCTGAGGGAG-3' (antisense).

Cell coupling

Three-day-old cultures of INS-1E cells were exposed for 24 h to the complete RPMI 1640 medium containing either 2 or 20 mmol/l glucose. The dishes were then transferred onto the heated (37 °C) stage of an inverted Zeiss ICM35 microscope, and individual cells were microinjected for 5 min, using glass pipettes containing 4% Lucifer Yellow CH (Sigma-Aldrich Chemie GmbH), in 10 mmol/l HEPES-buffered (pH 7.2) 150 mmol/l LiCl, and square negative pulses of 0.1 nA amplitude, 900 ms duration, and 0.5 Hz frequency (Charpantier *et al.* 2007, Bavamian *et al.* 2012). Coupling extent was calculated after each injection by scoring the number of cells containing the injected tracer. Coupling incidence was determined by calculating the percentage of injections resulting in the cell-to-cell transfer of the tracers. Coupling index was obtained by coupling extent X coupling incidence.

Statistical analyses

Data are expressed as mean $\pm$ S.E.M. Differences between means were assessed by Student's *t*-test. In experiments involving the comparison of more than two groups, ANOVA was used, followed by a *t*-test with Bonferroni correction. Real-time PCR data statistics were obtained by comparing, at each time point, the RNA levels observed in islets isolated from rats infused by NaCl to those observed in glucose-infused rats. Coupling data were analyzed by both ANOVA (extent) and the nonparametric χ^2 test (incidence). All computations were carried out using either the JMP software, version 3.2.2 (SAS Institute, Cary, NC, USA), or IBM SPSS Statistics 2 (SPSS, Inc.). The α -level of all tests was set at 0.05. Statistical significance was defined at a value of *P<0.05, **P<0.01, and ***P<0.001.

Results

Chronic hyperglycemia downregulates the *in vivo* expression of islet Cx36

The levels of plasma glucose and insulin were constant in the control rats that were infused with 0.9% NaCl for up to 48 h (Table 1), averaging 5.6 ± 0.1 mmol/l and 0.8 ± 0.05 ng/ml respectively in the 46 rats that were studied. In contrast, the companion rats, which were infused in parallel with glucose, showed significantly (*P*<0.001) higher levels of both plasma glucose and insulin at all time points (Table 1). However, while the levels of glucose increased with the duration of the glucose perfusion, those of insulin increased for the first 24 h of this perfusion, declining thereafter between 24 and 48 h (Table 1). The data validate the utility of the rat model in reproducing the major blood alterations of T2D *in vivo*. Quantitative

Table 1 Biological parameters evaluated in the NaCl- and glucose-infused rats, immediately prior to the pancreas sampling, which ended the *in vivo* part of the experiments. Data are mean $\pm$ S.E.M. values of the number of rats indicated in the right column

Time (h)	Infusion	Glycemia (mmol/l)	Insulinemia (ng/ml)	Body weight (g)	Number of rats
0	None	5.6 ± 0.1	0.8 ± 0.05	372 ± 6.4	46
6	NaCl	5.6 ± 0.1	0.7 ± 0.2	321 ± 16	5
	Glucose	$14.9 \pm 1.7^\dagger$	$9.4 \pm 1.2^\dagger$	344 ± 8	7
12	NaCl	6.0 ± 0.2	0.9 ± 0.1	368 ± 18	6
	Glucose	$12.6 \pm 0.6^\dagger$	$11.3 \pm 0.9^\dagger$	393 ± 14	7
24	NaCl	5.5 ± 0.2	1.1 ± 0.2	402 ± 13	4
	Glucose	$18.9 \pm 2.8^*$	$20.7 \pm 2.7^\dagger$	395 ± 23	6
48	NaCl	5.7 ± 0.3	1.0 ± 0.1	395 ± 20	5
	Glucose	$19.4 \pm 1.7^\dagger$	$7.9 \pm 1.1^\dagger$	399 ± 18	6

*P<0.01 and $^\dagger P$ <0.001 compared with the corresponding value in the rats perfused with 0.9% NaCl, as evaluated by ANOVA.

Figure 1

Chronic hyperglycemia differentially affected the transcription of different islet genes. Quantitative RT-PCR analysis showed that the expression of *Gjd2*, the transcript coding Cx36 (A), *Gapdh* (B), *Ins2* (C), *Icer1* (D), *Rab3a* (E), and *Rph3a* (F) mRNAs was not affected by NaCl perfusion. In contrast, sustained hyperglycemia progressively downregulated the expression of *Gjd2* (A) and *Ins2* (C) and of the two ICER-1 γ target genes *Rab3a* (E) and *Rph3a* (F). In the very same rats, the expression of *Gapdh* (B) and *Icer1* (D) was increased. Data are expressed after normalization to the mRNA levels of the housekeeping ribosomal *Rpl27* gene, whose expression was constant in these experiments. Normalized data are shown as mean $\pm$ S.E.M. of at least four rats per time point. * $P < 0.05$ and ** $P < 0.01$ vs the respective NaCl-infused group.

RT-PCR analysis of total RNA extracted from the islets isolated immediately after the end of each perfusion revealed that the levels of several transcripts, including those of *Gjd2*, *Ins2*, *Gapdh*, *Icer*, *Rab3a*, and *Rph3a*, were not significantly modified, throughout the experiment, in the control rats perfused with 0.9% NaCl (Fig. 1), which remained normoglycemic (Table 1). In contrast, significant changes in these transcripts were observed in the samples of the glucose-infused rats (Fig. 1), which became hyperglycemic (Table 1). Thus, while the transcripts of *Gjd2*, *Ins2*, *Rab3a*, and *Rph3a* decreased with the perfusion time, those of *Gapdh* and *Icer1* increased in parallel in the very same rats (Fig. 1). *Gapdh* mRNA expression is known to be upregulated by glucose (Roche *et al.* 1997) and, thus, was used as a positive control in the glucose-infused tissue

extracts. The data show that chronic hyperglycemia differentially regulates a variety of islet genes *in vivo* and specifically downregulates the transcriptional expression of *Gjd2*, which codes for Cx36. The concomitant increase in ICER-1/ICER-1 γ , a transcriptional repressor involved in the effect of palmitate on Cx36 expression (Allagnat *et al.* 2008), and the decrease in protein RAB3A and protein NOC2, two ICER-1/ICER-1 γ targets (Abderrahmani *et al.* 2006), suggested that this inducible early repressor may mediate the effect of high glucose concentrations on Cx36 expression.

Chronic exposure to high glucose concentrations downregulates the *in vitro* expression of INS-1E Cx36

To experimentally investigate this plausible molecular mechanism, we used the rat insulin-producing line INS-1E. In the presence of high glucose concentrations, these cells also reduced their native CX36 protein expression (Fig. 2A and B). This change was associated with a significant decrease in both the extent and the incidence of cell-to-cell coupling, as evaluated by the intercellular exchange of the gap junction tracer Lucifer Yellow (Table 2). Similar to islets, INS-1E cells exposed for a day to 20 mmol/l glucose decreased the expression of CX36, in a way which was inversely related to the parallel increase in the expression of nuclear ICER-1/ICER-1 γ , which was both time- and dose-dependent (Fig. 2A and B). In contrast, glucose had no effect on the expression of the CREM-1 protein. The glucose-mediated CX36 downregulation and overexpression of ICER-1/ICER-1 γ were both prevented in the presence of 5 μ mol/l of the cAMP-dependent protein kinase inhibitor H89 (Fig. 2C). With regard to the regulation of CX36 and ICER-1/ICER-1 γ expression, the data show that INS-1E cells mimicked *in vitro* the *in vivo* behavior of rat islets exposed to hyperglycemia.

ICER-1/ICER-1 γ regulates the expression of Cx36 in INS-1E cells

The promoters of the human and rodent *Gjd2* genes feature a highly conserved cAMP-response element (CRE) between bases –566 and –556, upstream of the transcription start site (Allagnat *et al.* 2005). To test whether this element mediates the effect of high glucose concentrations on Cx36 expression, we generated a plasmid expressing the reporter gene luciferase under the control of a 1 kb fragment of the human *GJD2* promoter, which contained the CRE (pGJD2). One day after transfection

Figure 2

High glucose levels time- and dose-dependently downregulate CX36 and induce ICER-1/ICER-1 γ expression in INS-1E cells. (A) Time-course analysis of total CX36 over tubulin and nuclear ICER-1/ICER-1 γ over CREM-1 protein levels in INS-1E cells exposed to 20 mmol/l glucose revealed a progressive, significant increase in the ICER-1/ICER-1 γ proteins and a parallel decrease in the CX36 protein. (B) The amplitude of these two changes was almost linearly dependent on the concentration of glucose tested during the 24-h duration of these experiments. (C) Exposure to 5 μ mol/l of PKA inhibitor H89 prevented the differential, glucose-dependent regulation of ICER-1/ICER-1 γ and CX36. The top part of each panel shows a representative immunoblot for CX36, ICER-1, ICER-1 γ , and the internal standards tubulin and CREM-1. The bottom part of each panel shows the densitometric analysis of three independent experiments. Data are mean $\pm$ s.e.m. values, expressed relatively to the corresponding internal standards (tubulin for CX36 and CREM-1 for ICER-1/ICER-1 γ). * P < 0.05, ** P < 0.01, and *** P < 0.001 vs the level evaluated at time 0 (A) or after a 24-h exposure to 5 mmol/l glucose (B and C).

with p*GJD2*, INS-E cells were incubated for an additional 24 h in the presence of either 5 or 20 mmol/l glucose and then processed to evaluate the activity of the *GJD2* promoter. Normalized luciferase measurements showed

that the *GJD2* promoter activity was 30% less (P < 0.01) in the cells exposed to 20 mmol/l glucose compared with those exposed to 5 mmol/l glucose (Fig. 3A). However, the effect of glucose was lost in cells transfected with a plasmid that contained the 1 kb *Gjd2* promoter in which the CRE had been point-mutated (p*GJD2* mut) (Fig. 3A). Then, INS-1E cells were co-transfected with a control empty vector (pCDNA3), a plasmid coding for ICER-1 (pICER-1), or an antisense construct (pAS-ICER), which reduces the endogenous expression of ICER-1/ICER-1 γ (Abderrahmani *et al.* 2006, Haeffliger *et al.* 2013). We observed that ICER-1 overexpression (Fig. 3B) reduced by 40% the activity of the native *Gjd2* promoter fragment in the cells exposed to 5 mmol/l glucose, but not in the cells exposed to 20 mmol/l glucose, and that this inhibitory effect was not observed in the cells transfected with p*GJD2* mut (Fig. 3A). Conversely, co-transfection with pAS-ICER resulted in a slightly increased luciferase activity of p*GJD2* in the cells exposed to 5 mmol/l glucose and restored the *GJD2* activity in the cells cultured in the presence of 20 mmol/l glucose (Fig. 3A). Altogether the data show that ICER-1/ICER-1 γ mediated the glucose effect on the *GJD2* promoter activity through the CRE.

Western blot analysis of INS-1E cells transiently transfected with the plasmid encoding ICER-1/ICER-1 γ further showed that ICER-1 overexpression (Fig. 3B) mimicked the downregulatory effect of 20 mmol/l glucose on CX36 protein levels (Fig. 3C), whereas the transfection of the antisense ICER construct, which prevented the overexpression of ICER-1/ICER-1 γ (Fig. 3B), abolished the glucose-induced CX36 downregulation (Fig. 3C).

To assess whether this regulation involved the direct binding of ICER-1/ICER-1 γ to the CRE of the *Gjd2* promoter, we immunoprecipitated the chromatin of INS-1E cells cultured in the presence of either 5 or 20 mM

Table 2 Dye coupling of INS-1E cells after a 24-h exposure to low and high glucose concentrations

Glucose (mmol/l)	Extent of coupling ^a	Incidence of coupling ^b	Coupling index ^c (a.u.)	Number of microinjections
2	2.7 $\pm$ 0.3	20 (71.4)	192.8	28
20	1.4 $\pm$ 0.1*	13 (40.6) [†]	56.9	32

^aData are mean $\pm$ s.e.m. values of the number of cells featuring labeling by Lucifer Yellow, indicated in the right column. * P < 0.001 compared with the value in the cells exposed to low glucose concentrations, as evaluated by ANOVA.

^bNumber and proportion of injections that did result in the intercellular transfer of dye. [†] P < 0.01, as evaluated by the nonparametric χ^2 test.

^cGiven by the average extent of coupling X the percentage incidence of coupling.

Figure 3

ICER-1/ICER-1 γ mediates the effect of glucose on Cx36 expression, by binding to a cAMP response CRE of the *Gjd2* promoter. (A) INS-1E cells were co-transfected with a reporter plasmid under the control of a 1 kb fragment of the human *GJD2* promoter (pGJD2) or the same fragment with a mutated CRE (pGJD2 mut), together with the control empty vector pCDNA3 or a plasmid allowing either the overexpression of ICER-1 (pICER-1) or the reduction of the endogenous expression of ICER-1/ICER-1 γ by an antisense effect (pAS-ICER-1). One day after transfection, the cells were incubated for 24 h in presence of either 5 mmol/l (black bars) or 20 mmol/l glucose (white bars) and assessed for luciferase activity. Results are *Firefly* luciferase activities normalized to *Renilla* luciferase activities and are shown as means $\pm$ s.e.m. of five independent experiments. * P < 0.05; ** P < 0.01 vs the pCDNA3-transfected cells, 5 mmol/l value. # P < 0.05 vs the pCDNA3-transfected cells, 20 mmol/l glucose. (B and C) INS-1E cells were transfected with an empty vector (pCDNA3) or plasmids allowing either the overexpression of ICER-1 (pICER-1) or the reduction of the endogenous ICER-1/ICER-1 γ levels (pAS-ICER-1). Forty-eight hours after transfection, the cells were cultured for an additional 24 h in the presence of either 5 or 20 mmol/l glucose. (B) Western blot analysis of ICER-1/ICER-1 γ and CREM-1 levels in nuclear extracts revealed the efficiency of the plasmid transfection. (C) Western blot analysis of CX36 and tubulin showed that ICER-1/ICER-1 γ

overexpression decreased CX36 expression, whereas the reduction of the repressor increased it and prevented the glucose-induced downregulation of the connexin. The top parts of panels (B) and (C) show representative Western blots; the lower parts show the densitometric evaluation of four independent experiments. Results were normalized to the internal controls (CREM-1 for ICER-1/ICER-1 γ and tubulin for CX36) and are shown as means $\pm$ s.e.m. of four independent experiments. * P < 0.05; ** P < 0.01, *** P < 0.001 vs 5 mmol/l glucose value. # P < 0.05, ** P < 0.01 vs the pCDNA3-transfected cells, 20 mmol/l value. (D) Chromatin was extracted from INS-1E cells cultured for 24 h in the presence of either 5 or 20 mmol/l glucose, with or without the PKA inhibitor H89 (5 μ mol/l). Fixed chromatin was immunoprecipitated using antibodies to CREM-1, REST, or c-Myc, and the fragment of the *Gjd2* promoter containing CRE was amplified by PCR. Quantitative RT-PCR of the immunoprecipitated promoter fragment demonstrated the binding of CREM-1, which was significantly greater in the cells exposed to 20 mmol/l glucose (D). This increase was abolished in the cells exposed to H89. No significant immunoprecipitation was observed in the cells exposed to 20 mmol/l glucose, when the chromatin was reacted with antibodies to either REST or c-Myc. Results are means $\pm$ s.e.m. of four independent experiments. ** P < 0.01 vs CREM-1, 5 mmol/l glucose value. ### P < 0.05 vs CREM-1, 20 mmol/l value.

glucose, using antibodies to CREM-1, which recognize ICER-1 γ . Antibodies against c-Myc, which have no putative binding site in the *Gjd2* promoter, and against REST, a repressor that is not expressed in β -cells (Martin *et al.* 2003), served as negative controls. The expected 385 bp amplicon, corresponding to bases -796 to -411 of the *Gjd2* promoter, was detected in the anti-CREM-1 immunoprecipitates, but not in the anti-Myc and the anti-REST ones (Fig. 3D). Quantitative PCR further revealed that the binding

of CREM-1 to the *Gjd2* promoter was significantly increased in the cells exposed to 20 mM glucose (Fig. 3D) and that this effect was blocked by treating the cells with H89 (Fig. 3D), which prevented the glucose-mediated overexpression of ICER-1/ICER-1 γ (Fig. 2C). The data indicate that the direct binding of ICER-1 to the CRE of the *Gjd2* promoter was necessary and sufficient to account for the downregulation of Cx36 expression observed after chronic exposure to high glucose levels.

Discussion

Chronic exposure to increased levels of environmental glucose is deemed as one of the most deleterious changes of the *milieu interieur*, which, by impacting on several tissues key to metabolic regulation, plays a major role in the development and maintenance of diabetes (Stolar 2010, Inzucchi *et al.* 2012). Specifically, high levels of glucose are thought to have a toxic impact on the function of the insulin-producing β -cells of pancreas, which play a prominent role in most forms of the disease (Robertson 2009, Ferrannini 2010, Poitout *et al.* 2010). Still, the molecular mechanisms linking hyperglycemia to β -cell dysfunction are still poorly understood and the subject of much debate (Robertson 2009, Ferrannini 2010, Poitout *et al.* 2010). We previously documented the *in vivo* role of the gap junction protein Cx36 in the biosynthetic and secretory functions of β -cells (Serre-Beinier *et al.* 2009), as well as in the resistance of these cells to conditions eliciting hyperglycemia (Klee *et al.* 2011). We also reported that the expression of this protein is downregulated under several *in vitro* and *in vivo* conditions mimicking in part a diabetic environment (Allagnat *et al.* 2005, Haefliger *et al.* 2013). At this point, however, it has not been established whether a sustained elevation of the circulating levels of glucose is alone sufficient to cause such a downregulation *in vivo* and, if so, by which mechanism.

To address these issues, we investigated a glucose-infused rat model. Of note, our model was designed so that the rats remained hyperglycemic throughout the experiment, in contrast with previous studies in which glycemia returned to control values 24 h after the beginning of infusion. In the latter, the moderate glycemia excursion allowed for β -cell adaptation via an increase in the β -cell mass, whereas in our model, the higher glycemic excursion did not allow for such compensation, resulting in β -cell failure. However, a few days of experimentally induced hyperglycemia is but an approximate model of the long-term *in vivo* conditions that presumably mediate the toxic effects of glucose on β -cell functions. Our purpose here was not to investigate the complex situation occurring in T2D, but rather to assess whether a sustained elevation of glucose would modulate Cx36 expression. In this perspective, the glucose infusion model appeared adequate to test this possibility *in vivo*. First, we observed that hyperglycemia rapidly stimulated the expression of the known glucose-responsive gene *Gapdh* (Roche *et al.* 1997), which validates the glucose infusion model. We further observed that chronic hyperglycemia inhibits within hours the expression of *Gjd2* mRNA, in parallel

with an induction of *Icer1*. This repressor is known to inhibit the expression of *Rab3a*, *Rph3al*, and *Ins2* (Abderrahmani *et al.* 2006). Accordingly, the transcription of these three genes was found to be decreased in the hyperglycemic animals, validating the glucose-infused rat model and stressing the central, *in vivo* repressor role of ICER-1/ICER-1 γ . The data extend the *in vitro* observations that ICER-1/ICER-1 γ is overexpressed in response to prolonged exposure to high glucose concentrations (Zhou *et al.* 2003, Abderrahmani *et al.* 2006) and are consistent with the report of increased levels of the repressor in the islets of diabetic GK rats (Inada *et al.* 1998), as well as in those of hypercholesterolemic *Apoe*^{-/-} mice (Haefliger *et al.* 2013) and of mice fed a high-fat diet (Allagnat *et al.* 2008).

To evaluate whether these coincidental changes are actually interrelated, we used the INS-1E rat insulin-producing cells in which glucose time- and dose-dependently decreases Cx36 expression (Allagnat *et al.* 2005), like it does in the primary islets. Under these conditions, we found that glucose also time- and dose-dependently stimulated ICER-1/ICER-1 γ expression, again like in the primary islets. Antisense blockade of this overexpression prevented the glucose-dependent downregulation of CX36, indicating that ICER-1/ICER-1 γ likely mediates the inhibitory effect of hyperglycemia on the expression of CX36. Further ChIP experiments revealed that ICER-1/ICER-1 γ directly binds to a CRE in the promoter of the Cx36 gene *Gjd2*, leading to a direct downregulation of Cx36 transcription, which ultimately decreases the levels of the cognate protein. We further showed that under these conditions, the CX36 protein downregulation results in a decrease in the extent and incidence of coupling between INS-1E cells.

In this study, we showed that hyperglycemia specifically induces an overexpression of ICER-1/ICER-1 γ and a concomitant downregulation of Cx36 in β -cells. Previous studies have documented the deleterious impact of prolonged ICER-1/ICER-1 γ overexpression on β -cell function and survival (Inada *et al.* 2004, 2005, Abderrahmani *et al.* 2006, Allagnat *et al.* 2008, Favre *et al.* 2011). However, little is known about the ICER-1 target genes responsible for the pro-apoptotic impact of this repressor. Together with our previous research (Allagnat *et al.* 2008, Favre *et al.* 2011, Haefliger *et al.* 2013), the present study has identified Cx36 as a relevant ICER-1/ICER-1 γ target. Given the requirement of a proper Cx36 signaling in insulin secretion and β -cell survival (Meda 2012), our data strongly suggest that Cx36 contributes to the deleterious sequence of events triggered by ICER-1/ICER-1 γ , which

presumably leads to glucose desensitization and β -cell failure in the pre-diabetic stage.

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.

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Author contribution statement

F A, P M, J-A H, and F R-J designed the project. F A, F R-J, A C, D C, P M, and J-A H performed the experiments. F A, P M, and J-A H wrote the manuscript.

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