Zn\(^{2+}\) is an important cofactor for insulin biosynthesis and storage in pancreatic \(\beta\)-cells. Correspondingly, polymorphisms in the \(SLC30A8\) gene, encoding the secretory granule Zn\(^{2+}\) transporter ZnT8, are associated with type 2 diabetes risk. Using a genetically engineered (FRET)-based sensor (eCALWY-4), we show here that elevated glucose time-dependently increases free cytosolic Zn\(^{2+}\) ([Zn\(^{2+}\)]\(_{cyt}\)) in mouse pancreatic \(\beta\)-cells. These changes become highly significant \((853 \pm 96 \text{ pM} \ versus \ 452 \pm 42 \text{ pM}, \ p < 0.001)\) after 24 h and are associated with increased expression of the Zn\(^{2+}\) importer family members \(Slc39a6, Slc39a7,\) and \(Slc39a8,\) and decreased expression of metallothionein 1 and 2. Arguing that altered expression of the above genes is not due to altered [Zn\(^{2+}\)]\(_{cyt}\), elevation of extracellular (and intracellular) [Zn\(^{2+}\)] failed to mimic the effects of high glucose.

By contrast, increases in intracellular cAMP prompted by 3-isobutyl-1-methylxanthine and forskolin partially mimicked the effects of glucose on metallothionein, although not ZnT, gene expression. Modulation of intracellular Ca\(^{2+}\) and insulin secretion with pharmacological agents (tolbutamide and diazoxide) suggested a possible role for changes in these parameters in the regulation of \(Slc39a6\) and \(Slc39a7\) but not \(Slc39a8,\) nor metallothionein expression. In summary, 1) glucose induces increases in [Zn\(^{2+}\)]\(_{cyt}\) which are then likely to facilitate the processing and/or the storage of insulin and its cocrystallization with Zn\(^{2+}\), and 2) these increases are associated with elevated expression of zinc importers. Conversely, a chronic increase in [Zn\(^{2+}\)]\(_{cyt}\) following sustained hyperglycemia may contribute to \(\beta\)-cell dysfunction and death in some forms of diabetes.

The total Zn\(^{2+}\) content of the mammalian pancreas is high, and these ions are chiefly localized to the islet \(\beta\)-cell (1). Correspondingly, Zn\(^{2+}\) plays an important role in both insulin synthesis and storage (2–3). Indeed, total Zn\(^{2+}\) concentrations reach millimolar levels in the interior of the dense-core granule (4), where two Zn\(^{2+}\) ions coordinate six insulin monomers to form the hexameric structure on which insulin crystals are based (5). The physiological importance of Zn\(^{2+}\) homeostasis within the \(\beta\)-cell in man has been emphasized recently by the finding that a non-synonymous polymorphism (rs13266634 C/T) in the \(SLC30A8\) gene, encoding the largely pancreatic, endocrine-restricted, granular zinc transporter ZnT8, is associated with an increased risk of type 2 diabetes (T2D)\(^2\) (6–9). Through the generation of mice bearing null alleles, we (10–11) and others (12) have shown previously that the absence of ZnT8 leads to the formation of amorphous \(\beta\)-cell secretory granules and mild glucose intolerance, consistent with a role for abnormal \(\beta\)-cell Zn\(^{2+}\) homeostasis in the pathogenesis of T2D (10–12).

A link between the zinc status of the body and diabetes has been known for many years (1). Several reports have shown that T2D patients display a marked decrease in total plasma Zn\(^{2+}\) and hyperzincuria compared with control subjects (13), suggesting the possibility that hyperglycemia may interfere with Zn\(^{2+}\) absorption in the kidney. Despite the consistent decrease in plasma Zn\(^{2+}\) observed across studies, the levels (total and free) of the ion within tissues (including muscle, kidney, and liver) are controversial (14–15). Reduced pancreatic Zn\(^{2+}\) has also been reported in a genetic model of T2D (16–18). However, no differences in the ultrastructural localization of Zn\(^{2+}\) were noted in \(\beta\)-cells from rats with monogenic (fa/fa Zucker) or more complex polygenic (Goto-Kakizaki) forms of diabetes (19).

Relatively little is known about how Zn\(^{2+}\) homeostasis is achieved in pancreatic \(\beta\)-cells and whether, and under what circumstances, changes in cytosolic Zn\(^{2+}\) ([Zn\(^{2+}\)]\(_{cyt}\)) may occur in these cells. When measured with a synthetic, intracellularly trappable probe (Zinquin), values for free [Zn\(^{2+}\)] recorded from whole pancreatic islets were decreased after infusion of high glucose (20). However, the subcellular localization of this particular probe (and others of the same class) (21) is unclear, as it tends to compartmentalize to undefined granular structures (11). The above findings (20) are thus difficult to interpret in terms of the alterations that may occur at the molecular level to perturb intracellular Zn\(^{2+}\) homeostasis as glucose concentrations increase.

The abbreviations used are: T2D, type 2 diabetes; [Zn\(^{2+}\)]\(_{cyt}\) cytosolic Zn\(^{2+}\); Mt, metallothionein(s); TPEN, N,N,N’-tetakis(2-pyridylmethyl) ethylenediamine; pyrithione, 2-mercaptopyridine N-oxide; K\(_{ATP}\), ATP-dependent K\(^{+}\) channel; VGCC, voltage-gated Ca\(^{2+}\) channel.

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1 To whom correspondence should be addressed: Section of Cell Biology, Faculty of Medicine, Imperial College London, Sir Alexander Fleming Building, Exhibition Road, London SW7 2AZ, UK. Tel: 44-20-759-43340; Fax: 44-20-579-43351; E-mail: g.rutter@imperial.ac.uk.
Glucose Regulates Cytosolic Zn\(^{2+}\) Homeostasis in β-Cells

Using a newly developed, genetically encoded FRET-based probe (eCALWY-4), we have recently measured free \([\text{Zn}^{2+}]_{\text{cyt}}\) in the β-cell line INS-1 (832/13) and found that it is buffered at 400 pm at low glucose concentrations (22). To tightly maintain this low free level of \(\text{Zn}^{2+}\), \(\beta\) and other cell types deploy a variety of mechanisms, including zinc transporters and binding proteins (23). There are two main classes of \(\text{Zn}^{2+}\) transporters. The \(\text{ZnT}\) family (coded by the \(\text{Slc30a}1–10\) genes) is thought to move the ion from the cytosol to intracellular compartments or into the extracellular space (24). The \(\text{Zip}\) (\(\text{Zrt-}\) and \(\text{Irt-like}\) proteins) family (coded by the \(\text{Slc39a}1–14\) genes), on the other hand, is responsible for increases in cytosolic \(\text{Zn}^{2+}\) (25). Evidence for the expression of these transporters in the pancreas is quite limited, although most studies agree on the fact that \(\text{Slc39a}6\) and \(\text{Slc39a}7\), among the \(\text{Zip}\), and \(\text{Slc30a}5\) and \(\text{Slc30a}8\), among the \(\text{ZnTs}\), are the most abundant transporters in pancreatic islets (26–27), with other evidence suggesting that \(\text{ZnT}1\) and \(\text{ZnT}4–9\) are also additionally expressed (11, 28). Importantly, \(\text{Zn}^{2+}\) can also be transported into β-cells via voltage-gated L-type \(\text{Ca}^{2+}\) channels (29) opened upon glucose-induced depolarization of the plasma membrane (30).

Metallothioneins (Mt) are redox-active \(\text{Zn}^{2+}\) binding proteins with a dual function, playing a role in both \(\text{Zn}^{2+}\) homeostasis and in the regulation of the cellular redox state. In the latter context, these proteins release \(\text{Zn}^{2+}\) in response to oxidative damage (31), a condition often found in the tissues of T2D patients, probably reflecting chronic hyperglycemia (32).

The expression in the β-cell of the above array of proteins involved in \(\text{Zn}^{2+}\) homeostasis argues for the importance of tight control of cytosolic \(\text{Zn}^{2+}\) levels. Indeed, \(\text{Zn}^{2+}\) plays a role not only in the defense of \(\beta\) and other cells against oxidative damage (as part of the CuZn superoxide dismutase (33) and metallothioneins (34)), but may mediate apoptosis if the free concentrations of the ion decrease or increase above a safe set-point (35–36).

In the present study we show firstly, by imaging free \([\text{Zn}^{2+}]_{\text{cyt}}\) in primary β-cells with eCALWY-4 (22), that glucose is able to induce a stable increase in the cytosolic concentration of these ions. Secondly, we show that high glucose leads to profound alterations in the expression of genes important for \(\text{Zn}^{2+}\) homeostasis in β-cells and explore the signaling mechanisms involved.

**EXPERIMENTAL PROCEDURES**

**Animals**—CD1 mice were housed with five mice per cage in a pathogen-free facility and were fed ad libitum with a standard mouse chow diet. Female mice were used at 10–12 week of age and were sacrificed by cervical dislocation as approved by the United Kingdom Home Office Animal Scientific Procedures Act, 1986.

**Reagents**—RPMI medium was obtained from Sigma. TRIZol reagent was from Invitrogen. \(\text{ZnCl}_2\), forskolin, 3-isobutyl-1-methylxanthine, tolbutamide, diazoxide, verapamil, N,N,N’,N’-tetraakis(2-pyridylmethyl) ethylenediamine (TPEN), and 2-mercaptopyridine N-oxide (pyrithione) were from Sigma.

**Islet Isolation and Dissociation into Single Cells**—Pancreatic mouse islets were prepared as described elsewhere (37). The islets were allowed to recover overnight in culture medium (RPMI supplemented with 10% (v/v) heat-inactivated FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin). Islets were dissociated by 10-min incubation in Hanks’-based enzyme-free cell dissociation buffer (GIBCO, Invitrogen), before centrifugation and gentle pipetting (37). Dissociated cells were plated onto 24-mm sterile coverslips and allowed to recover overnight.

**Generation of eCALWY-4-expressing Adenovirus and Infection of Primary Cells**—eCALWY-4 and eCALWY-NB (non-binding) fragments (22) were PCR-amplified (forward, CTCGAGGGCGCACATGAGGCCATAT; reverse, TCTAGA-GGCGCTTTACTGTCAGCT) and cloned into the pCR2.1-TA vector (Invitrogen) before centrifugation and gentle pipetting (37). The fragments were excised and cloned into plasmid pShuttle using XhoI/XbaI. Adenoviral constructs were generated as described elsewhere (38). Briefly, pShuttle-eCALWY constructs were digested with *Pme*I and electroporated into competent BJ5183-AD-1 cells. Recombined pADeasyI clones were screened, and positive clones were then digested with *PacI* and transfected into HEK293 cells for the generation of adenoviral particles. Primary islet cells were infected with eCALWY-4-expressing adenovirus (20–25 multiplicity of infection) for 4 h. The medium was then changed, and cells were allowed to express the protein for 48 h before imaging. Either 2 or 24 h before imaging, cells were incubated with different concentrations of glucose. These cultures typically contained 70–80% β-cells (39–40), and we confirmed the identity of individual β-cells by confocal microscopy (see Fig. 1B) in a limited number of experiments. Briefly, after infection with eCALWY-4-expressing adenovirus, primary cells were fixed in 3.7% paraformaldehyde and permeabilized in 0.1% Triton X-100 before immunostaining with a polyclonal anti-swine insulin antibody (1:200, DakoCytomation, Ely, UK) and an Alexa Fluor 568-coupled secondary antibody. Confocal imaging was performed as described elsewhere (40).

**Imaging Free Cytosolic Zn\(^{2+}\)**—Cells were washed twice in Krebs Hepes-bicarbonate buffer (140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH\(_2\)PO\(_4\), 0.2 mM MgSO\(_4\), 1.5 mM CaCl\(_2\), 10 mM Hepes (pH 7.4), 2 mM NaHCO\(_3\)), pre-equilibrated with 95:5 O\(_2\):CO\(_2\) and containing either 3 or 16.7 mM glucose. Zn\(^{2+}\) imaging was carried out as described by Vinkenborg et al. (22). Briefly, dissociated islet cells were maintained at 37 °C throughout with a heating stage (MC60, LINKAM, Scientific Instruments, Surrey, UK), and Krebs Hepes-bicarbonate buffer was perfused (1 ml/min) with additions as stated in Fig. 1C. 50 μM TPEN and 5 μM pyrithione were freshly prepared on each day of the experiments. Images were captured using an Olympus IX-70 wide-field microscope with a 40× oil immersion objective and an Imago charge-coupled device camera (Till Photonics, Graefiling, Germany) controlled by TILLvisION software (Till Photonics). For FRET measurements, a 455DRLP dichroic mirror (Chroma Technology) and two emission filters (Chroma Technology, D465/30 for cerulean and D535/30 for citrine), alternated by a filter changer (Lambda 10–2, Sutter Instruments), were used. Images were acquired at 1 Hz using a 100-ms exposure time and a 433-nm excitation wavelength. The fluorescence emission ratios were derived after subtracting background and calibrated for \([\text{Zn}^{2+}]_{\text{cyt}}\) as described under “Results.”
RNA Extraction and Quantitative Real-time PCR—For RNA extraction, 100 islets were incubated for 1 h in RPMI (20 islets/ml) containing 3 mM glucose and subsequently transferred into a 60-mm Petri dish with culture medium containing either 3 or 16.7 mM glucose. Total RNA was obtained using TRIzol reagent (Invitrogen) and treated with a DNA-free kit (Applied Biosystems, Warrington, UK). Total RNA was then reverse-transcribed into cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems). cDNA (equivalent to 30 ng of RNA) was subject to quantitative real-time PCR using Power SYBR Green master mix (Applied Biosystems) in a 7500 fast real-time PCR system (ABI Biosystems) and analyzed by the comparative Ct method. All real-time primers (supplemental Table 1) were generated using ABI Primer Express 3.0 software and were validated for specificity by dissociation curve (40).

Protein Extraction and Western Blotting (Immunoblotting) Analysis—For protein analysis, 250 (20 islets/ml of medium) islets were incubated for 1 h in RPMI containing 3 mM glucose before a further 24-h incubation with either 3 or 16.7 mM glucose. Islets were then washed twice in ice-cold PBS and lysed in ice-cold radioimmunoprecipitation assay buffer (50 mM Tris HCl (pH 8.0), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) before sonication. Protein was assayed with a BCA kit (Pierce). Total protein extracts (30 μg) were resolved by SDS-PAGE (12% v/v acrylamide) and transferred to PVDF membranes, followed by immunoblotting with rabbit polyclonal anti mLIV-1 (ZiP6) and anti mKE4 (ZiP7, both used 1:200, Ref. 41), mouse monoclonal anti-metallothioneins revealed no difference at high glucose (Fig. 3C). Western blotting with Slc39a7 antibodies showed a tendency to increase (853 ± 96 pm) compared with cells incubated at 3 mM glucose (452 ± 42 pm) (Fig. 1F). Although cytosolic Zn2+ concentrations were not significantly different after 2 h of incubation at high versus low glucose, a tendency toward an increase in the former case was observed (587 ± 101 pm at 16.7 mM glucose versus 496 ± 60 pm for cells incubated at 3 mM glucose, p = 0.431) (Fig. 1E), indicative of a time-dependent increase in [Zn2+]cyt in response to high glucose.

RESULTS

Elevated Glucose Promotes a Stable Increase in Cytosolic Free Zn2+ Concentrations in Primary β-Cells—Monitoring intracellular free Zn2+ in primary β-cells has so far only been achieved using synthetic chemical probes (11, 20). These sensors, however, suffer from limitations, such as a variable extent of compartmentalization into intracellular organelles, confounding both the measurements and their calibration. By generating an adenoviral expression construct based on our newly developed FRET-based Zn2+ probe, eCALWY-4 (22), we monitored cytosolic Zn2+ variations in primary β-cells and examined the time frames over which changes occurred. The ratio of citrine to cerulean emission (Fig. 1A) was plotted against time (C), and the minimum and maximum ratios were used to calculate the free [Zn2+]cyt using the following formula: [Zn2+] = Kd × (Rmax − R)/(R − Rmin), where the Kd for eCALWY-4 is 630 pm, Rmax represents the maximum fluorescence ratio (upon TPEN-mediated Zn2+ chelation), and Rmin is the minimum fluorescence ratio (obtained with 10 μM ZnCl2 in the presence of the Zn2+ ionophore, pyrithione, Ref. 22). Addition of TPEN or Zn2+ -pyrithione did not affect the ratio of a non-binding sensor variant (Fig. 1D). Cells were incubated for 1 h at 3 mM glucose before application of medium containing either 3 or 16.7 mM glucose for 2 or 24 h. The free [Zn2+]cyt was calculated by averaging the concentration of the ion 1 min before application of TPEN. After 24 h of treatment, cells incubated at 16.7 mM glucose displayed a substantial and significant (p < 0.001) [Zn2+]cyt increase (853 ± 96 pm) compared with cells incubated at 3 mM glucose (452 ± 42 pm) (Fig. 1F).

Regulation by Glucose of Genes Involved in Zn2+ Homeostasis—We hypothesized that the above variations in [Zn2+]cyt may, at least in part, reflect changes in the expression of genes known to regulate Zn2+ homeostasis in other cell types, namely ZiPs, ZnTs, and Mt. Thus, we measured the relative expression of the known mouse ZiPs (Slc30a7, ZnT (Slc30a) and metallothionein (Mt) genes in pancreatic islets at varying glucose concentrations and at two time points. Firstly, after 1-h incubation at 3 mM glucose, mouse islets were cultured in medium supplemented with either 3 or 16.7 mM glucose for 24 h. Culture with 16.7 versus 3 mM glucose led to a significant increase in Slc39a6 (2.4-fold), Slc39a7 (2.4-fold), and Slc39a8 (6.8-fold) mRNA levels but a decrease in Mt-1 (3.2-fold) and Mt-2 (4-fold) gene expression (Fig. 2A). The change in ZiP6 expression was also significantly reflected at the protein level, as demonstrated by Western blotting (immunoblotting) analysis (Fig. 3). ZiP7 protein expression showed a tendency to increase in response to high glucose (Fig. 3A), although the difference was very small and did not reach significance (B). We were unable to perform immunoblot analysis on ZiP7 because of the lack of a specific antibody against the mouse protein. Immunoblot analysis of metallothioneins revealed no difference at high versus low glucose concentrations (data not shown). In contrast, no changes in mRNA levels were observed in the expression of any of the tested Slc30a (ZnT) family members in response to variations in glucose concentration (Fig. 2A).

To test whether the changes in cytosolic free Zn2+ observed (Fig. 1F) upon high glucose challenge were the consequence or the cause of the above changes in gene expression (Fig. 2A), further qPCR analyses were performed after 2 h of treatment (well before the [Zn2+]cyt increase reached statistically significant level (Fig. 1E)). This analysis was restricted to the gene that showed a difference when treated with high versus low glucose after 24 h. Significant changes of between 1.8- and 2.1-fold were observed at this earlier time point in Slc39a7 and Mt-2 expression, respectively, whereas both Slc39a6 and Slc39a8 showed a tendency to
increase (and Mt-1 to decrease) at 16.7 versus 3 mM glucose (Fig. 2B).

The above findings suggested that the glucose-induced variations in gene expression were more likely to contribute to, rather than be the consequences of, changes in [Zn$^{2+}$]$_{cyt}$. To further explore this hypothesis, we exposed pancreatic islets to medium containing different glucose concentrations (as above), supplemented or not with 50 μM ZnCl$_2$ for 24 h, to increase intracellular free Zn$^{2+}$. To confirm that the concentration of extracellularly applied ZnCl$_2$ was indeed able to induce an increase in intracellular free Zn$^{2+}$, even in the absence of high glucose, we measured [Zn$^{2+}$]$_{cyt}$ after incubation of dissociated islets with 3 mM glucose in the presence of 50 μM ZnCl$_2$ for 24 h. As shown in Fig. 4A, extracellular ZnCl$_2$ elevated free [Zn$^{2+}$]$_{cyt}$ to a level comparable with that observed after incubation at high glucose concentrations (903 ± 89 pM versus 855 ± 138 pM). As anticipated (42), ZnCl$_2$ supplementation strongly induced the metallothionein genes while decreasing the expression of the Slc39a genes tested (Fig. 4B). Thus, the increase in cytosolic free Zn$^{2+}$ observed after 24 h of incubation with 16.7 mM glucose would appear likely to be a consequence of the changes in the expression of genes involved in controlling intracellular Zn$^{2+}$ fluxes and binding.

**Effect of L-type Voltage-gated Ca$^{2+}$ Channel (VGCC) Inhibition on Glucose-mediated [Zn$^{2+}$]$_{cyt}$ Increase**—It is known that influx of Zn$^{2+}$ from the extracellular space can be mediated by voltage-gated Ca$^{2+}$ channels, opened after glucose entry has caused K$_{ATP}$ channels closure and membrane depolarization (29). To test whether the increase of [Zn$^{2+}$]$_{cyt}$ reported here was via the glucose-induced opening of VGCCs, we measured intracellular Zn$^{2+}$ levels after dissociated islets were exposed for 24 h to 16.7 mM glucose alone or with the addition of 20 μM verapamil, a concentration known to block the VGCCs in pancreatic β-cells (43). Despite the presence of the VGCC blocker, high glucose concentrations were still able to induce an increase in free [Zn$^{2+}$]$_{cyt}$ to levels comparable with that found in the presence of 16.7 mM glucose alone (923 ± 137 pM for verapamil versus 855 ± 138 pM for the control condition, Fig. 5), suggesting that L-type Ca$^{2+}$ channels do not play a pivotal role in the rise of cytosolic Zn$^{2+}$ (Fig. 5).
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**FIGURE 2.** Effect of elevated glucose concentrations on the expression of the genes implicated in Zn\textsuperscript{2+} homeostasis in mouse pancreatic islets.

Mouse islets were incubated for 1 h at 3 mM glucose before incubation for 24 h (A) or 2 h (B) with either 3 mM (white bars) or 16.7 mM (black bars) glucose. Total RNA was extracted, and qPCR analysis of Slc39a1–12, Slc30a1–10, Mt-1, and Mt-2 was performed. The mRNA levels were normalized to those of a housekeeping gene (cyclophilin A). Bars represent mean ± S.E. (n = 4), *, p < 0.05.

Regulation of Slc39a6, Slc39a7, and Slc39a8, and Mt-1 and Mt-2, by Intracellular cAMP—To examine the potential signaling mechanisms responsible for glucose-evoked changes in gene expression, we first investigated whether increased cAMP levels, normally elevated in β-cells exposed to high glucose concentrations (44–46), might mimic the effect of the sugar. Suggesting this as a possibility, in silico examination of the mouse Slc39a6, Slc39a7, Slc39a8, Mt-1, and Mt-2 genes revealed consensus binding sites for the cAMP responsive element binding protein (47) in the promoter regions of all the genes examined, with the exception of Mt-2 (supplemental Table 2). Accordingly, pancreatic islets were incubated in 3, 10, or 16.7 mM glucose in the presence or absence of the adenylate cyclase activator forskolin and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (Fig. 6). Metallothionein expression was negatively modulated by elevation in cytosolic cAMP concentrations (3 mM glucose, mimicking the effect of high glucose). Correspondingly, a cAMP responsive element binding protein consensus binding site was apparent in the Mt-1 promoter (supplemental Table 2). The Mt-2 promoter, however, contained no such site, indicating that cAMP elevation might act via other mechanisms than those involving the cAMP responsive element binding protein. By contrast, elevated glucose (16.7 mM), in combination with increased cAMP, tended to decrease the expression of the Slc39a genes examined, a trend that was significant in the case of Slc39a7. At low glucose concentrations, on the other hand, cAMP elevations failed to increase Slc39a6–8 gene expression (Fig. 6). Thus, increases in intracellular cAMP appear unlikely to explain the enhanced expression of Slc39a6, Slc39a7, and Slc39a8 in response to high glucose.

Effect of Sulfonylureas and Diazoxide on Gene Expression—Insulin release and rebinding to β-cell insulin receptors is believed to be responsible for the regulation by glucose of several genes in this cell type (48–49). To determine whether such a mechanism may be involved in the regulation of genes involved in Zn\textsuperscript{2+} homeostasis, we stimulated or blocked insulin secretion pharmacologically using the K\textsubscript{ATP} channel modulators tolbutamide or diazoxide, respectively. The latter agent opens K\textsubscript{ATP} channels and thus prevents cell depolarization, elevation in cytosolic Ca\textsuperscript{2+}, and, therefore, insulin secretion (50). Pancreatic islets were incubated for 1 h at 3 mM glucose before further incubation with either 3 or 16.7 mM glucose for 24 h in the presence (or absence) of tolbutamide (51) or diazoxide (52).

Despite showing no significant difference compared with the control case (supplemental Fig. 1), incubation with tolbutamide abolished the effects of glucose on the expression of Slc39a6–8. As shown in supplemental Fig. 1, tolbutamide, which promotes cytosolic Ca\textsuperscript{2+} increases and insulin secretion even in the absence of glucose metabolism, tended to evoke an increase in Slc39a6 and Slc39a7 expression even at low glucose concentrations, thereby at least partially mimicking the effects of incubation in high glucose (supplemental Fig. 1). Similarly, diazoxide eliminated the effect of the sugar to stimulate Slc39a6 and
Slc39a7 gene expression (Fig. 7). Diazoxide also tended to decrease the expression of these genes, compared with the control case, at high glucose (Fig. 7). Surprisingly, perhaps, metallothionein gene expression was unchanged in the presence of either of these pharmacological agents. Slc39a8 expression was strongly up-regulated in the presence of diazoxide in combination with high glucose (Fig. 7).

Effect of Diazoxide on Cellular Stress and Free [Zn\(^{2+}\)]\(_{\text{cyt}}\) — Because Slc39a8 was very strongly up-regulated in the presence of high glucose plus diazoxide, we questioned whether this was due to an increase in cellular oxidative stress. Thioredoxin-interactin protein TxNIP participates in the control of the redox state of the cell by inhibiting thioredoxin (53) and has been identified as one of the most glucose-inducible genes in human and rodent islets (54). We therefore measured the levels of TxNIP expression in response to glucose in the presence or absence of diazoxide. As expected (54) and shown in Fig. 8A, high glucose increased the level of TxNIP expression by about 3.3-fold. However, when islets were incubated with high glucose in the presence of diazoxide, the expression of TxNIP was 25 times higher than in control condi-
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FIGURE 5. Effect of an L-type voltage-gated Ca\(^{2+}\) channel blocker on glucose-induced [Zn\(^{2+}\)]\(_{\text{cyt}}\) increase. Dissociated mouse islets were infected with eCALWY-4 expressing adenovirus before incubation with 3 or 16.7 mM glucose with or without 20 μM verapamil for 24 h before imaging on an Olympus IX microscope. [Zn\(^{2+}\)]\(_{\text{cyt}}\) was then calculated as explained under “Results” and in Fig. 1. Bars represent mean ± S.E. The number of cells imaged (n) on three different days of experiments is given in brackets under each bar. **, p < 0.01; ***, p < 0.001.

FIGURE 6. Effect of elevated intracellular cAMP levels on Slc39a6, Slc39a7, Slc39a8, Mt-1, and Mt-2 expression. Mouse islets were incubated for 1 h at 3 mM glucose before incubation at either 3, 10, or 16.7 mM glucose for 24 h in the presence (black bars) or absence (white bars) of 50 μM 3-isobutyl-1-methylxanthine and 10 μM forskolin. qPCR analysis of Slc39a6, Slc39a7, Slc39a8, Mt-1, and Mt-2 was performed. Values of expression were normalized to that of cyclophilin A. The fold changes compared with the values at 3 mM glucose were plotted. Bars represent mean ± S.E. (n = 4). **, p < 0.01; ***, p < 0.001.
other mammalian cells according to reports by many groups (55–57) but not others (58–59). Interestingly, we discovered that [Zn$^{2+}$]$_{\text{cyt}}$ substantially and progressively increases in response to prolonged (24-h) exposure to high glucose levels in mouse pancreatic $\beta$-cells. We also show that these changes begin as early as 2 h after the increase in glucose concentration, in line with earlier results that demonstrate Zn$^{2+}$ influx into mouse islet cells over shorter time frames (up to 15 min) using trappable chemical probes (29). Each of the above findings is in contrast, however, with earlier reports by Zalewski et al. (20), who observed a decrease in intracellular free Zn$^{2+}$ in islet cells in response to the sugar. However, these earlier measurements were performed using whole islets and a synthetic probe (Zinquin) that localizes, at least partly, to dense core granules and other membrane-bound organelles, where the free concentration of the ion may be much higher than in the cytosol (11). Thus, the decrease in apparent free Zn$^{2+}$ observed earlier may be due to confounding factors such as degranulation of the cells in response to glucose. Interestingly, when we blocked the L-type Ca$^{2+}$ channel with verapamil, we found that the increase in free [Zn$^{2+}$]$_{\text{cyt}}$ was comparable with that observed in the presence of high glucose alone, indicating that the flux of Zn$^{2+}$ via the VGCC plays, in this case, a minor role.

We also found that the increase in cytosolic free Zn$^{2+}$ concentration reported with eCALWy-4 was associated with increased expression of one of the key regulators of intracellular Zn$^{2+}$ homeostasis, namely ZiP6, both at the mRNA and protein level. Furthermore, we demonstrated an increase in ZiP7 and ZiP8 mRNA expression, although the lack of a suitable specific antibody against the latter precluded confirmation of such change at the protein level. ZiP7 immunoreactivity displayed a small increase at high glucose concentrations, but upon quantification, these changes were not significant. It should be emphasized that the analysis of gene expression was performed on whole pancreatic islets. These structures contain a mixed cell population ($\beta$-cells, $\alpha$-cells, and pancreatic polypeptide producing cells (60)) so that changes in gene expression may conceivably reflect alterations in any of these cell types. However, given that $\beta$-cells represent 60–80% of all islet cells, it is nonetheless likely that changes in the latter cell type predominate.

In line with our findings here, ZiP8 overexpression elicited by tissue necrosis factor-$\alpha$ (TNF-$\alpha$) induces an increase in intracellular Zn$^{2+}$, measured with FluoZin-3 in lung epithelial cells.
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FIGURE 8. Effect of the K\textsubscript{ATP} channel opener diazoxide on [Zn\textsuperscript{2+}]\textsubscript{cyt} and the expression of the marker of cellular stress, TxNIP. A, mouse islets were incubated for 24 h in the presence of either 3 or 16.7 mM glucose supplemented (black bars) or not (white bars) with 325 μM diazoxide before RNA extraction and analysis of TxNIP gene expression by quantitative real-time PCR. Values were normalized to the one of cyclophilin A, and the fold changes compared with control experiments at 3 mM glucose were plotted. Bars represent mean ± S.E. (n = 3). *, p < 0.05; **, p < 0.01; ***, p < 0.001. B, dissociated islets were infected with eCALWY-4-expressing adenovirus for 24 h and subsequently incubated with a further 24 h supplemented with 3 or 16.7 mM glucose in the presence or absence of 325 μM diazoxide before imaging on an Olympus IX microscope. [Zn\textsuperscript{2+}]\textsubscript{cyt} was then calculated as explained under “Results” and in the legend to Fig. 1. The number of cells imaged (n) on three different days of experiments is given in brackets under each bar. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

(61). Moreover, down-regulation of ZiP6 is associated with diminished free cytosolic Zn\textsuperscript{2+}, measured with the synthetic probe Newport green dichlorofluorescein, in dendritic cells (62). Both ZiP6 and ZiP8 localize to the plasma membrane of different cell types (61, 63, 64), although in T-cells (65), ZiP8 staining colocalized with lysosomal markers. ZiP7, on the other hand, is mainly localized to the Golgi apparatus (41), indicating that the observed increase in cytosolic free Zn\textsuperscript{2+} may result not only from enhanced influx of the ion from the extracellular space but also to release from (non secretory granule) intracellular compartments.

Surprisingly, perhaps, we did not observe any change in the expression of ZnT8/Slc30a8, the most abundant zinc transporter expressed in mouse pancreatic β-cells (10–11, 26), at high glucose concentrations, at least at the time points and under the culture conditions deployed here. Moreover, a recent report indicated that ZnT3 is expressed in pancreatic β-cells and that its expression increases in response to glucose (66). In contrast to these earlier findings but in line with Ref. 11, we failed here to observe detectable expression of the ZnT3-coding gene Slc30a3 or an increase in expression following glucose stimulation in primary β-cells. This might be explained by the fact that in the study by Smidt et al. (66), 4-week-old BALB/CA male mice were used, as opposed to the 12-week-old female CD1 mice used in the present study.

Despite the fact that we were unable to detect any changes at the protein level, the decrease in mRNA levels observed after 24 h at high glucose concentrations suggested a link between glucose metabolism and Mt expression.

A protective role for metallothioneins in diabetes has been suggested earlier from the observation that overexpression of these proteins in response to Zn\textsuperscript{2+} could attenuate streptozocin-induced diabetes in mice (67) and may reflect the fact that they are able to increase the oxidative defense of pancreatic β-cells. The observation that an elevation in cAMP induces a decrease in metallothionein expression suggests that this might be the pathway through which glucose modulates Mt expression in pancreatic β-cells. However, such a mechanism would be in contrast to what is observed in adipocytes, where high levels of cAMP are correlated with increased metallothionein expression (68).

A previous study using oligonucleotide microarrays (69) has shown that Slc39a6 and Mt-1a expression are altered at the mRNA level in rat islets after 96 h of culture at varying glucose concentrations, but earlier time points were not examined. This study extends these earlier results and begins to dissect the intracellular signaling mechanisms that lie behind the glucose-induced changes in the expression of key regulators of intracellular Zn\textsuperscript{2+} homeostasis in the pancreatic β-cell. A model summarizing the changes that we report here is presented in Fig. 9. Whether altered zinc importer and metallothionein expression reflect alterations in the transcription of the corresponding genes or in mRNA (or protein) stability, or combinations of the above, were not addressed in this study and will require further detailed investigation in future.

Nonetheless, our findings implicate an unsuspected role for cAMP in the control by glucose of Mt-1 and Mt-2 mRNA expression, whereas an increase in cytosolic (and possibly nuclear) Zn\textsuperscript{2+} is unlikely to be involved in (and would rather be expected to antagonize) the regulation by glucose of these genes. On the other hand, a role for cAMP in the control of Slc39a6–8 expression by glucose seems unlikely, at least on the basis of the impact of a single large increase in the concentrations of intracellular cAMP, as achieved here by pharmacological means. Moreover, our results suggest that glucose might act via different mechanisms in each case. Either tolbutamide or diazoxide (which respectively mimic and reverse the effects of glucose on intracellular free Ca\textsuperscript{2+}), thus affecting insulin secre-
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![Proposed model of the effect of glucose on the regulation of Zn^{2+} homeostasis in pancreatic β-cells.](image)

Although high glucose induces insulin secretion, it also known to induce oxidative stress, as observed by others (76) and assessed by us with the measurement of expression of TXNIP. This change results in the suppression of the reducing activity of thioredoxin, which is expected in turn to prompt in an increase in reactive oxygen species generation and will therefore contribute further to oxidative stress. Enhanced oxidative...
stress might then prompt metallothioneins to release bound Zn\(^{2+}\), therefore increasing [Zn\(^{2+}\)]\(_{cyt}\) even more. This, together with the lowering of metallothionein expression, may further compromise β-cell survival at later time points. Although further studies are needed to assess the relative importance of changes in cytosolic [Zn\(^{2+}\)] in the short and long term, it seems reasonable to speculate that tight control of free Zn\(^{2+}\) concentrations in β-cells may provide a means to prevent the decline in functional β-cell mass, which characterizes T2D (77).

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REFERENCES

1. Scott, D. A., and Fisher, A. M. (1938) J. Clin. Invest. 17, 725–728
2. Emdin, S. O., Dodson, G. G., Cutfield, J. M., and Cutfield, S. M. (1980) Diabetologia 19, 174–182
3. Hutton, J. C., Bailleys, E. M., Rhodes, C. J., Rutherford, N. G., Arden, S. D., and Guest, P. C. (1990) Biochem. Soc. Trans. 18, 122–124
4. Foster, M. C., Leapman, R. D., Li, M. X., and Atwater, I. (1993) Biophys. J. 64, 525–532
5. Hill, C. P., Dauter, Z., Dodson, E. J., Dodson, G. G., and Dunn, M. F. (1991) Biochemistry 30, 917–924
6. Saxena, R., Voight, B. F., Lyssenko, V., Burtt, N. P., de Bakker, P. I., Chen, H., Roix, J. J., Kathiresan, S., Hirschhorn, J. N., Daly, M. J., Hughes, T. E., Groop, L., Altshuler, D., Almgren, P., Florez, J. C., Meyer, J., Ardlie, K., Bengtsson Bostroem, K., Isomaa, B., Lettre, G., Lindblad, U., Lonnqvist, J., Laurila, E., Melander, O., Newton-Cheh, C., Nilsson, P., Orho-Melander, M., Rastam, L., Speliotes, E. K., Taskinen, M. R., Tuomi, T., Guiducci, C., Berglund, A., Carlson, J., Gianniny, L., Hackett, R., Hall, L., Holmquist, J., Laurila, E., Sjogren, M., Sterner, M., Surti, A., Svensson, M., Tewhey, R., Blumenstiel, B., Parkin, M., Defelice, M., Barry, R., Brodeur, W., Comarata, J., Chia, N., Fava, M., Gibbons, J., Handsaker, B., Healy, C., Nguyen, K., Gates, C., Sougnez, C., Gage, F., Dizzone, M., Gabriel, S. B., Chin, G. W., Ma, Q., Parikh, H., Richardson, D., Richee, D., and Purcell, S. (2007) Science 316, 1331–1336
7. Scott, L. I., Mohlke, K. L., Bonnycastle, L. L., Will, C. J., Li, Y., Duren, W. L., Emdin, S. O., Stringham, H. M., Chines, P. S., Jackson, A. U., Prokunina-Olsson, L., Ding, C. I., Swift, A. J., Narisu, N., Hu, T., Pruim, R., Xiao, R., Li, X. Y., Conneely, K. N., Riebow, N. L., Sprau, A. G., Tong, M., Bozym, R. A., Chimienti, F., Giblin, L. J., Gross, G. W., Korichneva, I., Li, Y., Huang, L., Kirschke, C. P., Zhang, Y., and Yu, Y. Y. (2005) Biochemistry 44, 725–728
8. Southon, S., Cherrf, Z., Wright, A. J., and Fairweather-Tait, S. J. (1988) Br. J. Nutr. 60, 499–507
9. Sondergaard, L. G., Stoltenberg, M., Flyvbjerg, A., Brock, B., Schmitz, O., Danscher, G., and Runghy, J. (2003) APNIS 111, 1147–1154
10. Zalewski, P. D., Millard, S. H., Forbes, I. J., Kapaniris, O., Slavotinek, A., Betts, W. H., Ward, A. D., Lincoln, S. F., and Mahadevan, I. (1994) J. His-tochem. Cytochem. 42, 877–894
11. Sensi, S. L., Ton-Tha, D., Weiss, J. H., Rotha, A., and Gee, K. R. (2003) Cell Calcium 34, 281–284
12. Vinkenborg, J. L., Nicolson, T. J., Bellomo, E. A., Koay, M. S., Rutter, G. A., and Merkx, M. (2009) Nat. Meth. 6, 737–740
13. Liu, J. P., and Cousins, R. J. (2004) Annu. Rev. Nutr. 24, 151–172
14. Palmiter, R. D., and Huang, L. (2004) Pflugers Arch. 447, 744–751
15. Guerinot, M. L. (2000) Biochim. Biophys. Acta 1465, 190–198
16. Chimenti, F., Favier, A., and Seve, M. (2005) Biochem. 58, 313–317
17. Kambe, T., Narita, Y., Hymaguchi-Iwai, Y., Hirose, J., Amano, T., Sugia, N., Sasaki, R., Mori, K., Iwana, T., and Nagao, M. (2002) J. Biol. Chem. 277, 19049–19055
18. Wijesekara, N., Chimenti, F., and Wheeler, M. B. (2009) Diabetes Obes. Metab. 11, 202–214
19. Gyulkhandanyan, A. V., Lee, S. C., Bikopoulos, G., Dai, F., and Wheeler, M. B. (2006) J. Biol. Chem. 281, 9361–9372
20. Ashcroft, F. M., Proks, P., Smith, P. A., Ammal, C., Bovkist, K., and Rorsman, P. (1994) J. Cell. Biochem. 55, 54–65
21. Maret, W., and Kretzal, A. (2007) Mol. Med. 13, 371–376
22. Low, B. B., and Shulman, G. I. (2005) Science 307, 384–387
23. Crouch, R. K., Gandy, S., Patrick, J., Reynolds, S., Buse, M. G., and Simson, I. A. (1984) Exp. Mol. Path. 41, 377–383
24. Chen, H., Carlson, E. C., Pellet, L., Moritz, J. T., and Epstein, P. N. (2001) Diabetes 50, 2040–2046
25. Bozym, R. A., Chimenti, F., Giblin, L. J., Gross, G. W., Korichneva, I., Li, Y., Libert, S., Maret, W., Parviz, M., Frederickson, C. J., and Thompson, R. B. (2010) Exp. Biol. Med. 235, 741–750
26. Kim, B. J., Kim, Y. H., Kim, S., Kim, J. W., Koo, J. Y., Oh, S. H., Lee, M. K., Kim, K. W., and Lee, M. S. (2000) Diabetes 49, 367–372
27. Ravier, M. A., and Rutter, G. A. (2005) Diabetes 54, 1789–1797
28. Luo, J., Deng, Z. L., Luo, X., Tang, N., Song, W. X., Chen, J., Sharff, K. A., Loo, H. H., Haydon, R. C., Kinzler, K. W., Vogelstein, B., and He, T. C. (2007) Nat. Protoc. 2, 1236–1247
29. Elayat, A. A., el-Naggar, M. M., and Tahir, M. (1995) J. Anat. 186, 629–637
30. Meur, G., Qian, Q., da Silva Xavier, G., Pullen, T. J., Tsuboi, T., McKinnon, J. A., Fletcher, L., Tavare, J. M., Hughes, S., Johnson, P., and Rutter, G. A. (2011) J. Biol. Chem. 286, 13647–13656
31. Huang, L., Kirschke, C. P., Zhang, Y., and Yu, Y. Y. (2005) J. Biol. Chem. 280, 15456–15463
32. Radtke, F., Heuchel, R., Georgiev, O., Hergersberg, M., Gariglio, M., Dembic, Z., and Schaffner, W. (1993) EMBO J. 12, 1355–1362
33. Szollosi, A., Nenquin, M., and Henquin, J. C. (2010) Br. J. Pharmacol. 159, 669–677
34. Charles, M. A., Fanska, R., Schmid, F. G., Forsman, P. H., and Grodskey, G. M. (1973) Science 179, 569–571
35. Dyachok, O., Idevall-Hagren, O., Søgetorp, J., Tian, G., Wuttke, A., Arrieu-Bergan, C., Akusjärvi, G., Gylfe, E., and Tengholm, A. (2008) Cell Metab. 8, 26–37
36. Grill, V., and Cerasi, E. (1973) FEBS Lett. 33, 311–314
37. Janssion, D., Ng, A. C., Fu, A., Depatie, C., Al Azzabi, M., and Serecat,
Glucose Regulates Cytosolic Zn$^{2+}$ Homeostasis in β-Cells

R. A. (2008) Proc. Natl. Acad. Sci. U.S.A. 105, 10161–10166

48. Da Silva Xavier, G., Qian, Q., Cullen, P. J., and Rutter, G. A. (2004) Biochem. J. 377, 149–158

49. Leibiger, B., Moede, T., Schwarz, T., Brown, G. R., Köhler, M., Leibiger, I. B., and Berggren, P. O. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 9307–9312

50. Bryan, J., and Aguilar-Bryan, L. (1997) Curr. Opin. Cell Biol. 9, 553–559

51. Sánchez-Alvarez, R., Paíno, T., Herrero-González, S., Medina, J. M., and Tabernero, A. (2006) Glia 54, 125–134

52. Ma, Z., Portwood, N., Brodin, D., Grill, V., and Björklund, A. (2007) Diabetes 56, 1095–1106

53. Nishiyama, A., Matsui, M., Iwata, S., Hirota, K., Masutani, H., Nakamura, H., Takagi, Y., Sono, H., Gon, Y., and Yodoi, J. (1999) J. Biol. Chem. 274, 21645–21650

54. Bryan, J., and Aguilar-Bryan, L. (1997) Curr. Opin. Cell Biol. 9, 553–559

55. Sanchez-Alvarez, R., Páinio, T., Herrero-González, S., Medina, J. M., and Tabernero, A. (2006) Glia 54, 125–134

56. Ma, Z., Portwood, N., Brodin, D., Grill, V., and Björklund, A. (2007) Diabetes 56, 1095–1106

57. Nishiyama, A., Matsui, M., Iwata, S., Hirota, K., Masutani, H., Nakamura, H., Takagi, Y., Sono, H., Gon, Y., and Yodoi, J. (1999) J. Biol. Chem. 274, 21645–21650

58. Shalev, A., Pise-Masison, C. A., Radonovich, M., Hoffmann, S. C., Hirshberg, B., Brady, J. N., and Harlan, D. M. (2002) Endocrinology 143, 3695–3698

59. Krezel, A., and Maret, W. (2006) J. Biol. Inorg. Chem. 11, 1049–1062

60. Bryan, J., and Aguilar-Bryan, L. (1997) Curr. Opin. Cell Biol. 9, 553–559

61. Krezel, A., and Maret, W. (2006) J. Biol. Inorg. Chem. 11, 1049–1062

62. Li, Y., and Maret, W. (2009) Exp. Cell Res. 315, 2463–2470

63. Chowanadisai, W., Lönnnerdal, B., and Kelleher, S. L. (2008) Brain Res. 1199, 10–19

64. Leibiger, B., Moede, T., Schwarz, T., Brown, G. R., Köhler, M., Leibiger, I. B., and Berggren, P. O. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 9307–9312

65. Taylor, K. M., Morgan, H. E., Johnson, A., Hadley, L. J., and Nicholson, R. I. (2003) Biochem. J. 375, 51–59

66. Bryan, J., and Aguilar-Bryan, L. (1997) Curr. Opin. Cell Biol. 9, 553–559

67. Aydemir, T. B., Liuzzi, J. P., McClellan, S., and Cousins, R. J. (2009) J. Leukoc. Biol. 86, 337–348

68. Trayhurn, P., Duncan, J. S., Wood, A. M., and Beattie, J. H. (2000) Horm. Metab. Res. 32, 542–547

69. Bensellam, M., Van Lommel, L., Overbergh, L., Schuit, F. C., and Jonas, J. C. (2009) Diabetologia 52, 463–476

70. da Silva Xavier, G., Varadi, A., Ainscow, E. K., and Rutter, G. A. (2000) J. Biol. Chem. 275, 36269–36277

71. Michael, D. J., Ritzel, R. A., Haataja, L., and Chow, R. H. (2006) Diabetes 55, 600–607

72. Knoch, K. P., Bergert, H., Borgonovo, B., Saege, H. D., Altkrüger, A., Verkade, P., and Solimena, M. (2004) Nat. Cell Biol. 6, 207–214

73. Wijesekara, N., Dai, F. F., Hardy, A. B., Giglou, P. R., Bhattacharjee, A., Koshkin, V., Chimienti, F., Gaisano, H. Y., Rutter, G. A., and Wheeler, M. B. (2010) Diabetologia 53, 1656–1668

74. Carroll, R. J., Hammer, R. E., Chan, S. J., Swift, H. H., Rubenstein, A. H., and Steiner, D. F. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 8943–8947

75. Ghafghazi, T., McDaniel, M. L., and Lacy, P. E. (1981) Diabetes 30, 341–345

76. Bindokas, V. P., Kuznetsov, A., Sreenan, S., Polonsky, K. S., Roe, M. W., and Philipson, L. H. (2003) J. Biol. Chem. 278, 9796–9801

77. Kahn, S. E. (2001) Int. J. Clin. Pract. Suppl. 123, 13–18