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Research Article **Endocrinology**

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Chronic activation of a designer G_q-coupled receptor improves β cell function

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Type 2 diabetes (T2D) has emerged as a major threat to human health in most parts of the world. Therapeutic strategies aimed at improving pancreatic β cell function are predicted to prove beneficial for the treatment of T2D. In the present study, we demonstrate that drug-mediated, chronic, and selective activation of β cell G_q signaling greatly improve β cell function and glucose homeostasis in mice. These beneficial metabolic effects were accompanied by the enhanced expression of many genes critical for β cell function, maintenance, and differentiation. By employing a combination of in vivo and in vitro approaches, we identified a novel β cell pathway through which receptor-activated G_q leads to the sequential activation of ERK1/2 and IRS2 signaling, thus triggering a series of events that greatly improve β cell function. Importantly, we found that chronic stimulation of a designer G_q-coupled receptor selectively expressed in β cells prevented both streptozotocin-induced diabetes and the metabolic deficits associated with the consumption of a high-fat diet in mice. Since β cells are endowed with numerous receptors that mediate their cellular effects via activation of G_q-type G proteins, our findings provide a rational basis for the development of novel antidiabetic drugs targeting this class of receptors.

Introduction

Type 2 diabetes (T2D) has emerged as a major threat to human health in most parts of the world (1). A key pathophysiological feature of T2D is the inability of pancreatic β cells to release sufficient amounts of insulin to overcome peripheral insulin resistance and maintain normal glucose homeostasis (2, 3). The inability of β cells to adjust insulin release to maintain blood glucose levels within a physiological range is due to multiple impairments in β cell function combined with a significant reduction in β cell mass (2, 4). Consequently, therapeutic strategies aimed at improving β cell function and/or maintaining (or increasing) β cell mass are likely to prove beneficial for the treatment of T2D.

Like most other cell types, pancreatic β cells express various G protein–coupled receptors (GPCRs) on their cell surface. The individual receptors are linked to distinct families of heterotrimeric G proteins (G_s, G_i, or G_q), which have multiple effects on β cell function (5). Recently, new antidiabetic drugs have been approved that act by stimulating the activity of the G_s-coupled glucagon-like peptide-1 (GLP-1) receptor, which is expressed by pancreatic β cells as well as other cell types (5–7). Several studies have shown that drug-dependent activation of this G_s-coupled receptor improves β cell function and triggers increases in β cell mass, at least under certain experimental conditions (6, 7).

Pancreatic β cells also express several GPCRs that are linked to G_q-type G proteins, including the M_3 muscarinic acetylcholine receptor and GPR40, a receptor for free fatty acids (5, 8). Following acute activation of this class of receptors, activated $G\alpha_q$ subunits promote the PLCβ-mediated generation of 2 second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG stimulates the activity of different isoforms of PKC,

while IP_3 triggers the release of Ca^{2+} from endoplasmic reticulum stores. These signaling events are known to play key roles in promoting insulin secretion (5, 9).

Unfortunately, G_q-linked GPCRs expressed by β cells are also present in many other tissues or cell types, and receptor-subtype selective agonists useful for in vivo studies are not available in most cases. For this reason, it has been difficult to assess whether drug-dependent, chronic activation of β cell G_q signaling may prove beneficial for the treatment of T2D. To overcome these difficulties, we recently generated a mutant mouse strain (β-Rq mice) that expresses a G_q-coupled designer GPCR (referred to as Rq; ref. 10) selectively in pancreatic β cells (please note that Rq represents the rat version of the hM3Dq designer receptor; ref. 11). In this previous study, we verified that Rq is not expressed in the brain (hypothalamus/cerebral cortex). Importantly, the Rq designer receptor does not bind any endogenous ligand but can be selectively activated by clozapine-N-oxide (CNO), a compound that is otherwise pharmacologically inert (10, 11). The availability of this new mouse model offers the unique opportunity to selectively activate β cell G_q signaling in vivo in a drug-dependent fashion. Such CNO-sensitive designer GPCRs are also referred to as designer receptors exclusively activated by designer drug (DREADDs) or second-generation receptors activated solely by synthetic ligand (RASSLs) (11, 12).

In an initial study, we focused on the metabolic effects observed after acute CNO treatment of β-Rq mice (10). We found that acute stimulation of β cell G_q signaling led to enhanced insulin release, reduced blood glucose levels, and improved glucose tolerance (10).

The primary goals of the present study were to explore the potential therapeutic relevance of persistently activating β cell G_q signaling in mouse disease models and to elucidate the molecular mechanisms underlying the observed beneficial metabolic effects. We found that chronic CNO treatment of β-Rq mice resulted in striking improvements in β cell function, including the upregulation

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of many genes critical for β cell function, maintenance, and replication. Importantly, persistent stimulation of β cell G_q signaling effectively prevented streptozotocin-induced (STZ-induced) diabetes and greatly reduced the detrimental metabolic effects caused by the consumption of a high-fat diet (HFD).

To explore the molecular pathways underlying these beneficial metabolic effects, we carried out a series of in vitro and in vivo experiments. These studies led to the identification of a signaling pathway through which activation of β cell G_q triggers enhanced expression and function of IRS2, a cytoplasmic signaling protein that mediates many of the cellular actions of insulin and IGF1. Our results strongly support the concept that IRS2 plays a key role in mediating improved β cell function caused by chronic activation of β cell G_q signaling. These findings should stimulate the development of classes of antidiabetic drugs aimed at enhancing signaling through G_q-coupled receptors expressed by pancreatic β cells.

Results

Chronic activation of β cell G_q signaling lowers blood glucose levels and increases pancreatic insulin content and β cell mass. To examine the in vivo metabolic consequences of chronically stimulating a G_q-coupled receptor selectively expressed in pancreatic β cells, we treated 7- to 8-week-old male WT or β-Rq mice maintained on regular mouse chow for 4 weeks with daily i.p. injections of either CNO (1 mg/kg) or saline. CNO treatment had no significant effect on the body weight of β-Rq and WT mice (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI66432DS1). However, the CNO-treated β-Rq mice displayed a progressive reduction of blood glucose levels during the 4-week observation period (Figure 1A). This blood glucose–lowering effect was accompanied by an approximately 2-fold increase in plasma insulin levels in the CNO-treated β-Rq mice, as compared with the 3 control groups (saline- or CNO-treated WT mice and saline-treated transgenic mice; Figure 1B). Moreover, pancreatic insulin content was selectively increased in the CNO-treated β-Rq mice (Figure 1C). In contrast, pancreatic glucagon levels were similar in all 4 groups of mice (Figure 1D).

Strikingly, β-Rq mice that had been treated with CNO for 4 weeks displayed an approximately 2- to 3-fold increase in β cell mass as compared with the 3 control groups (Figure 1E), consistent with a previous report (10). This effect was accompanied by a significant increase in mean islet size (islet density remained unaffected by CNO treatment; Supplemental Table 1). To determine whether the CNO-dependent increase in β cell mass observed with β-Rq mice was due to enhanced β cell replication, we carried out morphometric studies using pancreatic slices double-stained for insulin and BrdU incorporation. We found that the number (%) of BrdU-positive (BrdU⁺) β cells was approximately 3-fold higher in islets from CNO-treated β-Rq mice as compared with the 3 control groups (Figure 1F). In contrast, the number of apoptotic β cells, measured as percentage of TUNEL⁺ insulin-staining cells, did not differ significantly among the 4 groups of mice (Figure 1G). There were no obvious changes in islet architecture among the different experimental groups (data not shown). These observations suggest that drug-mediated activation of a G_q-coupled receptor selectively expressed in pancreatic β cells leads to an increase in β cell mass, most likely by stimulating β cell proliferation.

Chronic activation of β cell G_q signaling enhances the expression of many genes critical for β cell function and proliferation. As outlined in the previous paragraph, chronic activation of β cell G_q signaling resulted in increases in pancreatic insulin content, β cell mass, and β cell pro-

liferation. To explore the potential molecular mechanisms underlying these effects, we carried out quantitative RT-PCR (qRT-PCR) studies to examine the expression levels of a series of genes important for β cell function, maintenance, and proliferation. For these studies, we used total RNA prepared from islets of β-Rq mice and their WT littermates that had been treated for 4 weeks with daily i.p. injections of either CNO (1 mg/kg) or saline. Interestingly, islets prepared from CNO-treated β-Rq mice showed significantly increased expression levels of the genes coding for preproinsulin (*Ins2*) and proprotein convertases 1 and 2 (*Pcsk1* and *Pcsk2*, respectively) as compared with the 3 control groups (Figure 2A). Since Pcsk1 and Pcsk2 are critical for the conversion of preproinsulin to insulin, these data support the concept that chronic activation of β cell G_q signaling promotes the synthesis of insulin. The transcript levels of *Glut2* and pyruvate carboxylase (*Pcx*), 2 genes critical for β cell function, were also selectively increased in islets prepared from CNO-treated β-Rq mice (Figure 2A). The expression levels of the glucokinase, glucagon, and acetyl-CoA carboxylase 2 genes were not significantly different among the 4 experimental groups (data not shown). Notably, however, *Irs2* expression was dramatically increased (by ~5- to 10-fold) in islets derived from the CNO-treated β-Rq mice as compared with the 3 control groups (Figure 2B). Consistent with these observations, we previously reported that CNO treatment (1 μM CNO for 3 hours) of isolated pancreatic islets prepared from β-Rq mice resulted in significant increases in *Irs2*, *Ins2*, *Pcsk1*, and *Pcsk2* gene expression levels (10).

We also examined the expression levels of several transcription factors that are known to play key roles in β cell differentiation, maintenance, and replication, including *MafA*, *Pdx1*, Neurogenin 3 (*Ngn3*), *Nkx6.1*, and *NeuroD1*. We found that the transcript levels of these 5 genes were selectively increased in islets prepared from CNO-treated β-Rq mice (Figure 2C). Given the known roles of these transcription factors in promoting β cell differentiation, function, and growth, it is likely that enhanced expression of these genes contributes to the increase in β cell mass and proliferation caused by chronic activation of β cell G_q signaling in vivo.

Chronic stimulation of β cell G_q signaling increases IRS2 protein expression and function. The pronounced increase in *Irs2* expression in islets from CNO-treated β-Rq mice was the most striking effect that we observed at the transcriptional level (Figure 2B). To determine whether these changes at the RNA level also increased IRS2 protein levels, we carried out a series of immunoblotting studies. We first treated β-Rq mice and their WT littermates (7- to 8-week-old males) for 4 weeks with CNO (CNO was mixed into drinking water at a concentration of 0.25 mg/ml). Mice consuming regular drinking water served as control animals. In β-Rq mice, chronic consumption of CNO water affected blood glucose levels, plasma insulin concentrations, and islet gene expression levels in a fashion similar to that observed after daily i.p. injections of CNO (Supplemental Figure 2). At the end of the CNO treatment period, we carried out Western blotting studies using lysates prepared from mouse pancreatic islets. In agreement with the gene expression data, we found that IRS2 protein levels were significantly higher (by ~2- to 3-fold) in islets derived from CNO-treated β-Rq mice (Figure 2, D and E). On the other hand, the expression levels of IRS1, another major IRS protein expressed by pancreatic islets, were similar in CNO- and saline-treated β-Rq mice (Figure 2, D and E).

To determine whether increased IRS2 expression led to enhanced downstream signaling in pancreatic islets of CNO-treated β-Rq mice, we studied the expression levels of phosphorylated Akt and
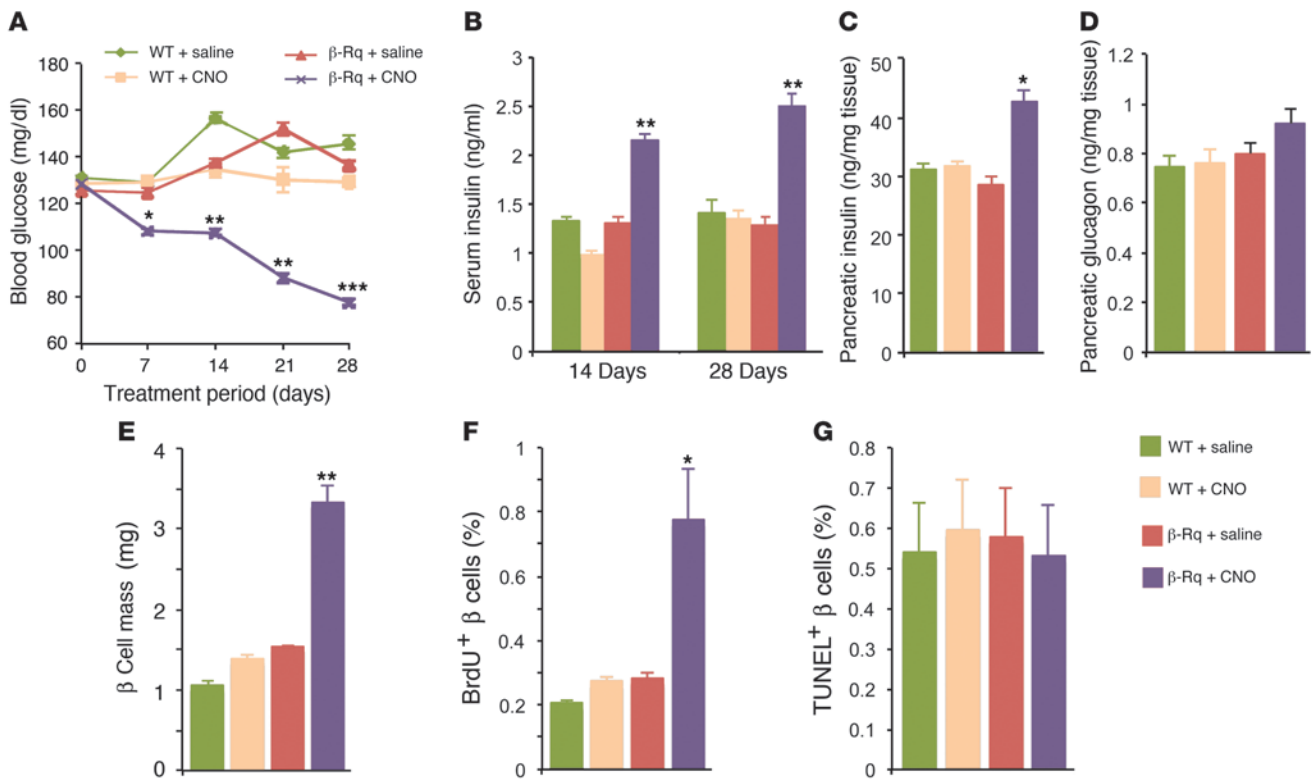
Figure 1
Chronic activation of G_q signaling in β cells in vivo increases β cell function and mass. All experiments were performed with β-Rq transgenic mice and WT littermates (males) maintained on regular mouse chow. Mice received daily i.p. injections of either CNO (1 mg/kg) or saline for 4 weeks (CNO treatment was initiated when the mice were 7 to 8 weeks old). For **C**–**G**, measurements were carried out at the end of the 4-week treatment period. (**A**–**D**) Progressive reduction of blood glucose levels (**A**; freely fed mice), elevated plasma insulin levels (**B**; freely fed mice), increased pancreatic insulin content (**C**), and unchanged pancreatic glucagon levels (**D**) in CNO-treated β-Rq mice. (**E**–**G**) Increased β cell mass (**E**), increased rate of β cell proliferation (**F**; percentage of insulin-positive cells that are BrdU+), and unchanged rate of β cell apoptosis (**G**; percentage of insulin-positive cells that are TUNEL+) in CNO-treated β-Rq mice. Data are expressed as mean ± SEM (**A** and **B**, $n = 6$ per group; **C**–**G**, $n = 3$ or 4 per group). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, as compared with the 3 control groups.

ERK1/2. Akt and ERK1/2 are key kinases that mediate many of the important cellular functions of activated IRS proteins (13). Total Akt and ERK1/2 expression levels did not differ significantly between the 2 groups of mice (CNO- vs. saline-treated transgenic mice; Figure 2, D and E). However, chronic CNO treatment of β-Rq mice greatly enhanced (by ~2- to 3-fold) the phosphorylation of Akt and ERK1/2. Taken together, these data support a model in which chronic activation of β cell G_q signaling promotes IRS2 expression and function.

Activation of a G_q-coupled receptor expressed by an insulinoma cell line promotes Irs2 expression in an ERK1/2-dependent fashion. To explore the mechanism that links enhanced β cell G_q signaling to increased IRS2 expression, we used a rat insulinoma cell line (INS1-M3) as a model system (14). This cell line expresses moderate levels of the M_3 muscarinic receptor subtype, a prototypic G_q-coupled receptor (15). Treatment of these cells with increasing concentrations of oxotremorine-M (OXO-M), a hydrolytically stable muscarinic agonist, had no significant effect on intracellular cAMP levels (Figure 3A). In contrast, forskolin, a direct activator of adenylyl cyclase, promoted cAMP formation with high potency and efficacy (Figure 3A). As expected, OXO-M treatment of INS1-M3 cells resulted in concentration-dependent increases in intracellular calcium levels (Figure 3B), a response that is characteristic for G_q-coupled receptors.

As observed with islets obtained from CNO-treated β-Rq mice (Figure 2B), prolonged stimulation (5 hours) of INS1-M3 cells with OXO-M (100 μM) led to a robust increase in *Irs2* mRNA expression (Figure 3C). This effect could be blocked by treatment of cells with the muscarinic antagonist, atropine (10 μM), indicative of the involvement of M_3 muscarinic receptors. The magnitude of the OXO-M–induced increase in *Irs2* mRNA expression was similar to that observed following treatment of INS1-M3 cells with forskolin (10 μM), an activator of adenylyl cyclase (Figure 3C).

It has been shown that stimulation of INS1-M3 cells with a muscarinic agonist promotes the release of insulin (14). Thus, to exclude the possibility that the OXO-M–induced increase in *Irs2* transcript levels was an indirect effect caused by secreted insulin acting on INS1-M3 cells, we measured insulin levels in the incubation medium at the end of the OXO-M treatment period (56.1 ng/ml ± 0.3; $n = 3$). We then incubated INS1-M3 cells with 55 ng/ml of insulin for 5 hours (instead of OXO-M) and performed qRT-PCR studies to assess *Irs2* gene expression. Under these experimental conditions, insulin treatment of INS1-M3 cells had no significant effect on *Irs2* transcript levels (Supplemental Figure 3).

We next used several kinase inhibitors to map the pathway that links M_3 receptor activation to increased *Irs2* expression in INS1-M3 cells. As expected, the PKA inhibitor H-89 (10 μM) had
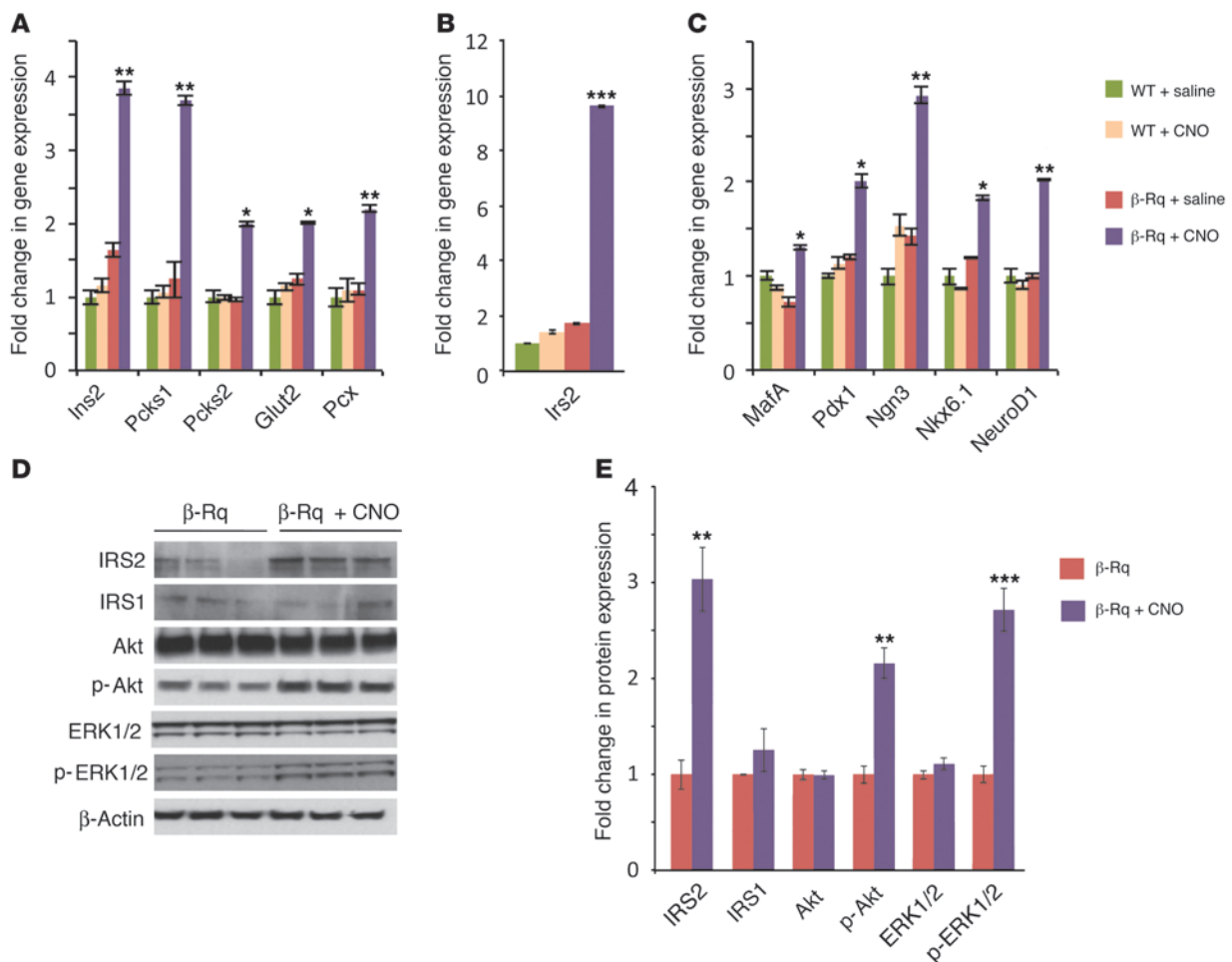
Figure 2
Chronic activation of G$_q$ signaling in β cells in vivo promotes the expression of many genes critical for β cell function. Subgroups of mice of each genotype (β-Rq transgenic mice and WT littermates, 7- to 8-week-old males) were chronically treated with CNO for 4 weeks. For **A–C**, CNO was administered via daily i.p. injections (1 mg/kg; control mice received saline). For **D** and **E**, CNO was administered via drinking water (0.25 g/ml). Experiments were carried out at the end of the 4-week treatment period. (**A–C**) Enhanced expression of key β cell genes in islets prepared from CNO-treated β-Rq mice, including genes critical for insulin synthesis (**A**), *Irs2* (**B**), and several important β cell transcription factors (**C**). Islet gene expression was studied by real-time qRT-PCR using total islet RNA. Data were normalized relative to the expression of 18S rRNA and are presented as fold change in gene expression compared with saline-treated WT mice. (**D** and **E**) Enhanced protein expression of IRS2, p-Akt, and p-ERK1/2 in islet lysates prepared from CNO-treated β-Rq mice. Representative Western blots are shown in **D**, while panel **E** provides a summary of all Western blotting experiments. In each individual experiment, protein levels were expressed relative to those found in non–CNO-treated β-Rq mice. Data are expressed as mean ± SEM (**A–C**, *n* = 3 per group; **D** and **E**, *n* = 6 [each lane corresponds to islet protein pooled from 2 different mice]). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, as compared with the 3 control groups (**A–C**) or vs. non–CNO-treated β-Rq mice (**E**).

no significant effect on OXO-M–induced *Irs2* expression (Figure 3D). In contrast, OXO-M–stimulated *Irs2* expression was greatly reduced or abolished by pharmacological inhibitors of PLC (U73122, 10 μM), PKC (GF109203X, 2.5 μM), and MEK1/2 (U0126, 10 μM) (Figure 3D). These data are in good agreement with a model in which M₃ receptor–mediated activation of G$_q$ leads to increased *Irs2* expression via sequential activation of PLC (PLCβ), PKC, and ERK1/2.

To provide additional experimental support for this model, we employed siRNA technology to knock down the expression of ERK1/2 in INS1-M3 insulinoma cells (Supplemental Figure 4). Treatment with ERK1/2 siRNA had no significant effect on cell viability (Supplemental Figure 5). Strikingly, OXO-M–stimulated *Irs2* expression was virtually abolished following knockdown of

ERK1/2 expression (Figure 3E). This observation strongly suggests that G$_q$-mediated stimulation of *Irs2* expression is mediated via a pathway that requires the activation of ERK1/2.

CNO treatment of isolated islets from β-Rq mice stimulates Irs2 expression and ERK1/2 phosphorylation. To investigate whether a similar pathway was operational in β-Rq mice, we carried out a series of studies with freshly isolated pancreatic islets from β-Rq mice. Consistent with the results obtained with INS1-M3 cells, prolonged incubation (3 hours) of β-Rq islets with CNO (1 μM) resulted in a robust increase in *Irs2* mRNA (Figure 3F), as reported in a previous study (10). This response was abolished by treatment of islets with inhibitors of PLC, PKC, or MEK1/2 (Figure 3F). Moreover, in agreement with the INS1-M3 cell data, ERK1/2 phosphorylation was only detectable in β-Rq islets that
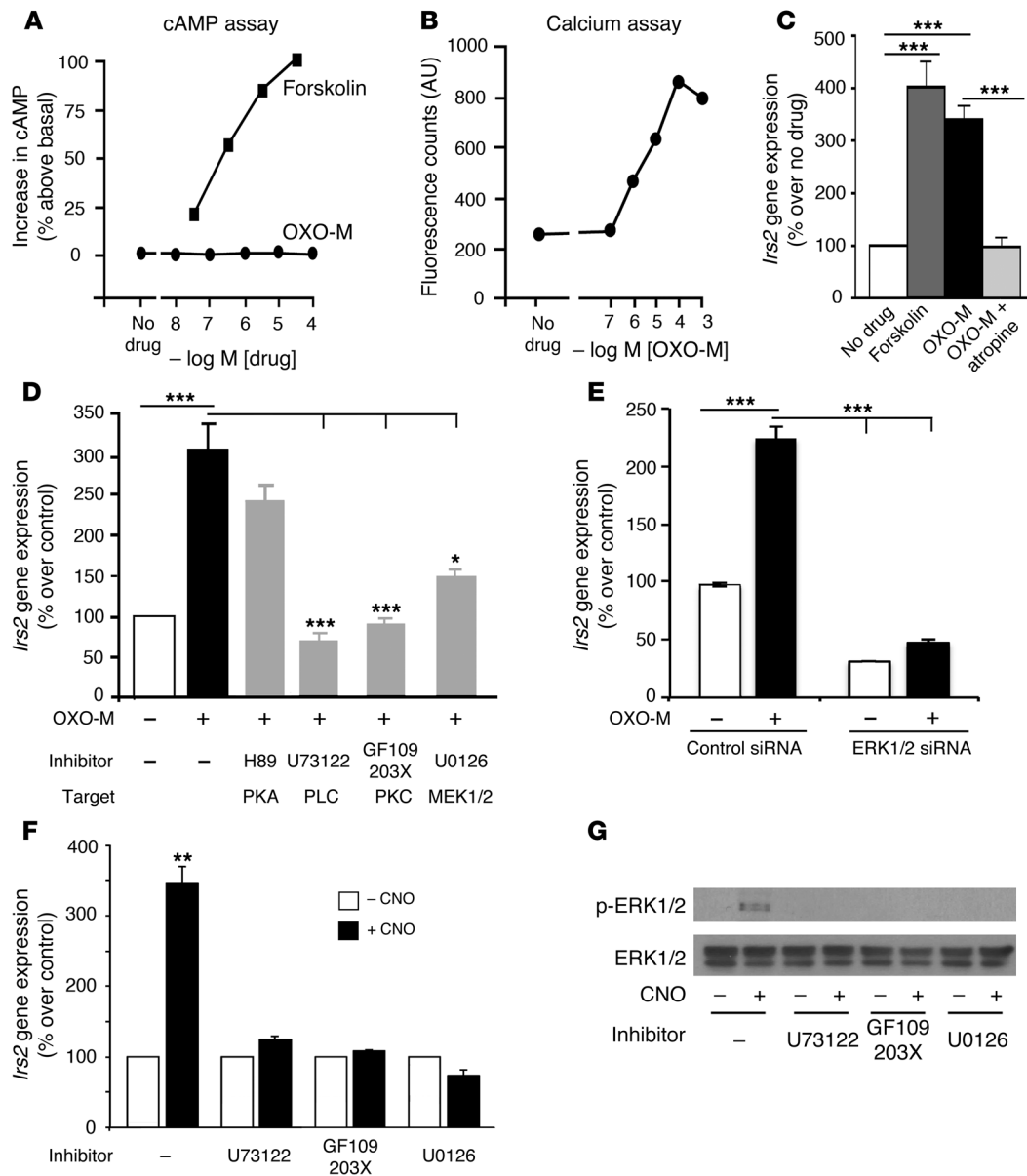
Figure 3

M3 muscarinic receptor/Rq-mediated increases in *Irs2* expression are PLC, PKC, and ERK1/2 dependent. (**A** and **B**) Treatment of INS1-M3 insulinoma cells with OXO-M, a muscarinic agonist, has no effect on intracellular cAMP levels (**A**), but leads to pronounced increases in intracellular calcium concentrations (**B**). The curves shown are representative of 3 independent experiments. (**C**) Incubation of INS1-M3 cells with OXO-M (100 μM; 5 hours) results in a robust increase in *Irs2* mRNA expression that can be blocked by atropine (10 μM), a muscarinic antagonist. Forskolin (10 μM), a direct activator of adenylyl cyclase, causes a response of similar magnitude. (**D**) Pharmacological inhibitors of PLC (U73122, 10 μM), PKC (GF109203X, 2.5 μM), and MEK1/2 (U0126, 10 μM) prevent or greatly reduce the OXO-M–induced (100 μM) increase in *Irs2* expression in INS1-M3 cells. (**E**) siRNA-mediated knockdown of ERK1/2 expression abolishes the OXO-M–induced (100 μM) increase in *Irs2* expression in INS1-M3 cells. (**F** and **G**) Incubation of isolated islets prepared from β-Rq mice with CNO (1 μM) results in a robust increase in *Irs2* mRNA levels (3 hours CNO incubation; **F**) and ERK1/2 phosphorylation in islet lysates (10 minutes CNO incubation; **G**). These effects were not observed after treatment of islets with inhibitors of PLC, PKC, or MEK1/2 (see above; also see the legend to Figure 2 for additional experimental details). Data represent mean ± SEM of 2 or 3 independent experiments (**G** shows representative Western blots). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

had been treated with CNO (1 μM for 10 minutes; Figure 3G). Under our experimental conditions, no ERK1/2 phosphorylation was observed with β-Rq islets that had not been exposed to CNO or with islets that had been incubated with inhibitors of PLC, PKC, or MEK1/2, either in the absence or the presence of CNO (Figure 3G).

The beneficial metabolic effects caused by chronic activation of β cell G_q signaling are IRS2 dependent. To test the hypothesis that the beneficial metabolic effects observed after chronic activation of β cell G_q signaling in β-Rq mice were dependent on the presence (activity) of IRS2, we carried out additional in vivo experiments. Specifically, we generated IRS2-deficient mice (*IRS2*^{−/−} mice; ref. 16) that carried the
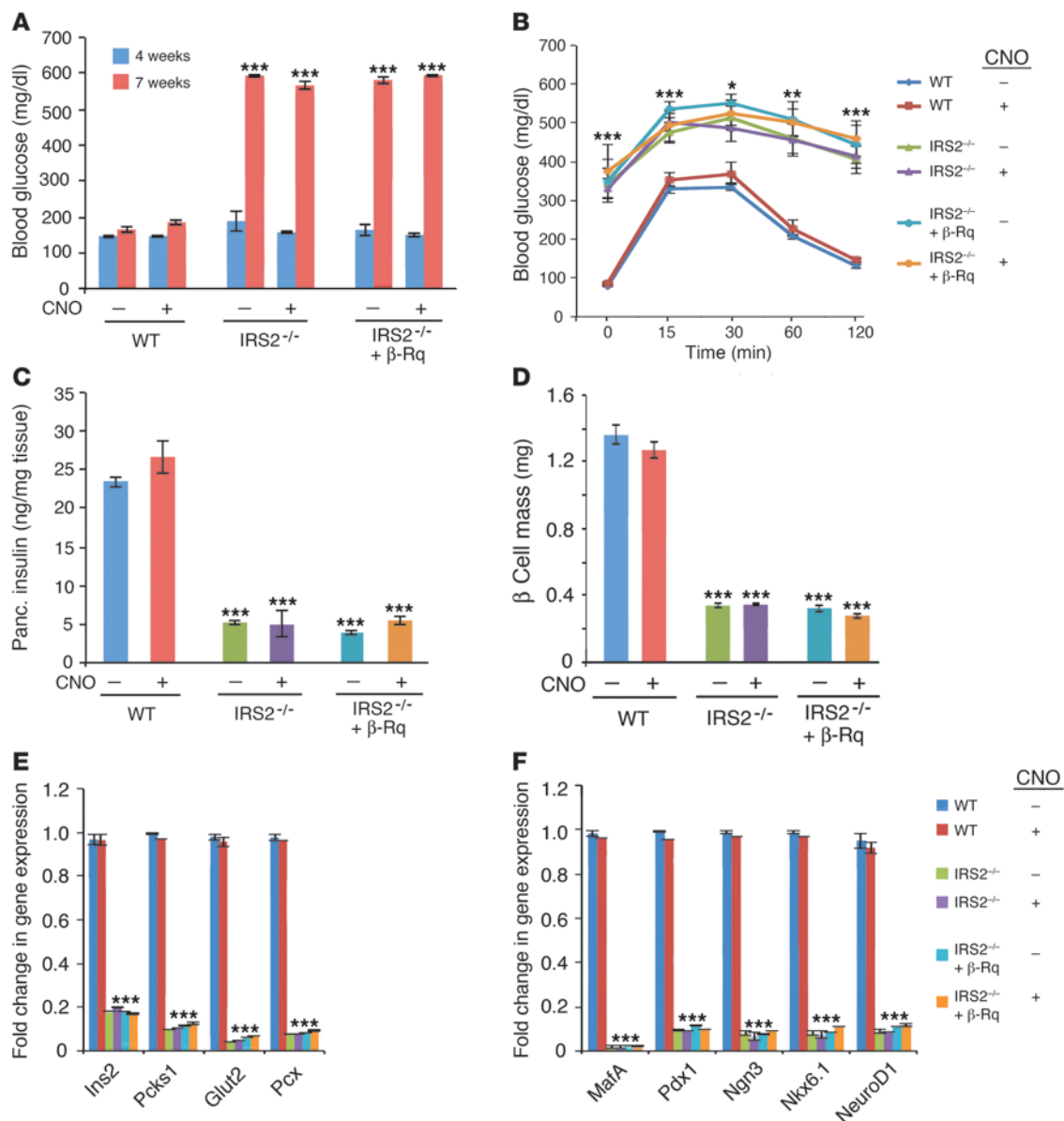
Figure 4
The beneficial metabolic effects caused by activation of β cell G_q signaling require the presence of IRS2. All studies were carried out with male WT mice, IRS2-deficient mice (*IRS2^(-/-)*), and *IRS2^(-/-)* mice carrying the β-Rq transgene (IRS2^(-/-) + β-Rq). When the mice were 4 weeks old, a subgroup of mice from each genotype received CNO via drinking water (0.25 g/ml) for 3 weeks. (**A–D**) CNO treatment of *IRS2^(-/-)* + β-Rq mice has no significant effect on hyperglycemia (**A**), glucose intolerance (**B**; IGTT, 2 mg/g glucose i.p.), reduced pancreatic (panc.) insulin content (**C**), and decreased β cell mass (**D**) caused by IRS2 deficiency. (**E** and **F**) CNO treatment of *IRS2^(-/-)* + β-Rq mice fails to increase the expression levels of β cell genes downregulated by IRS2 deficiency, including genes critical for β cell function (**E**) and key β cell transcription factors (**F**). Gene expression was studied by real-time qRT-PCR analysis of total islet RNA. Data were normalized relative to the expression of 18S rRNA and are presented as fold change in gene expression versus untreated WT mice. CNO effects were assessed using 7-week-old male mice. Data are expressed as mean ± SEM (*n* = 4 per group). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, as compared with the corresponding 4-week-old mice (**A**) or WT mice (**B–F**).

β-Rq transgene and then subjected the resulting double-mutant mice (*IRS2^(-/-)*/β-Rq mice) to a series of metabolic tests. For control purposes, WT mice and *IRS2^(-/-)* mice were analyzed in parallel.

Consistent with previous findings (16), 7-week-old *IRS2^(-/-)* mice, similarly to the *IRS2^(-/-)*/β-Rq mice double-mutant mice, displayed a pronounced diabetes phenotype, characterized by marked hyperglycemia and glucose intolerance (Figure 4, A and B) and prominent reductions in pancreatic insulin content and β cell mass (Figure 4,

C and D). Previous studies have shown that these metabolic deficits are caused primarily by the lack of IRS2 in pancreatic β cells (17–19).

To examine whether activation of β cell G_q signaling could prevent or ameliorate the detrimental metabolic effects caused by IRS2 deficiency, we treated *IRS2^(-/-)*/β-Rq double-mutant mice chronically with CNO via drinking water (0.25 mg/ml). CNO treatment was started when the mice were 4 weeks old and appeared metabolically normal. When the *IRS2^(-/-)*/β-Rq mice double-mutant
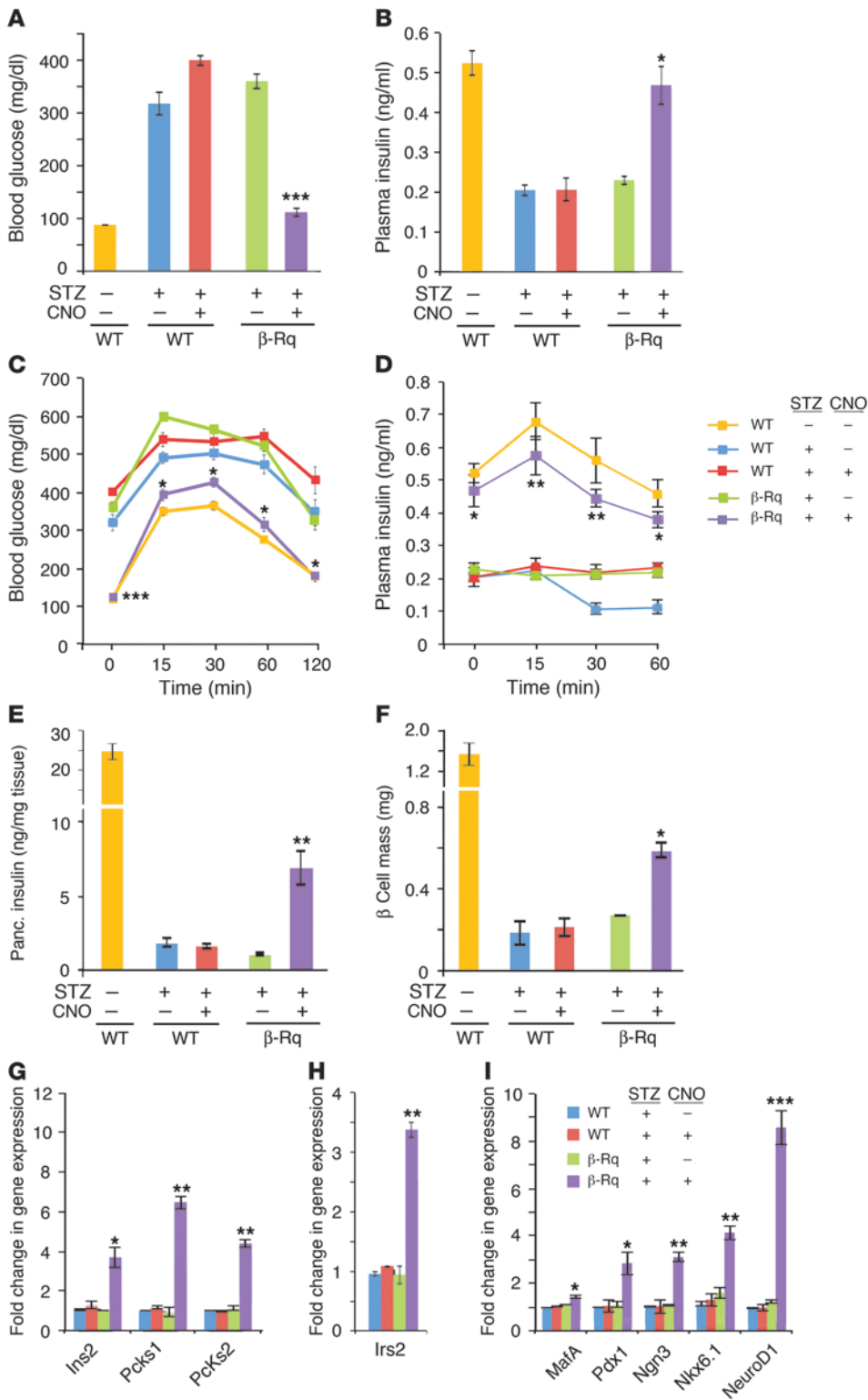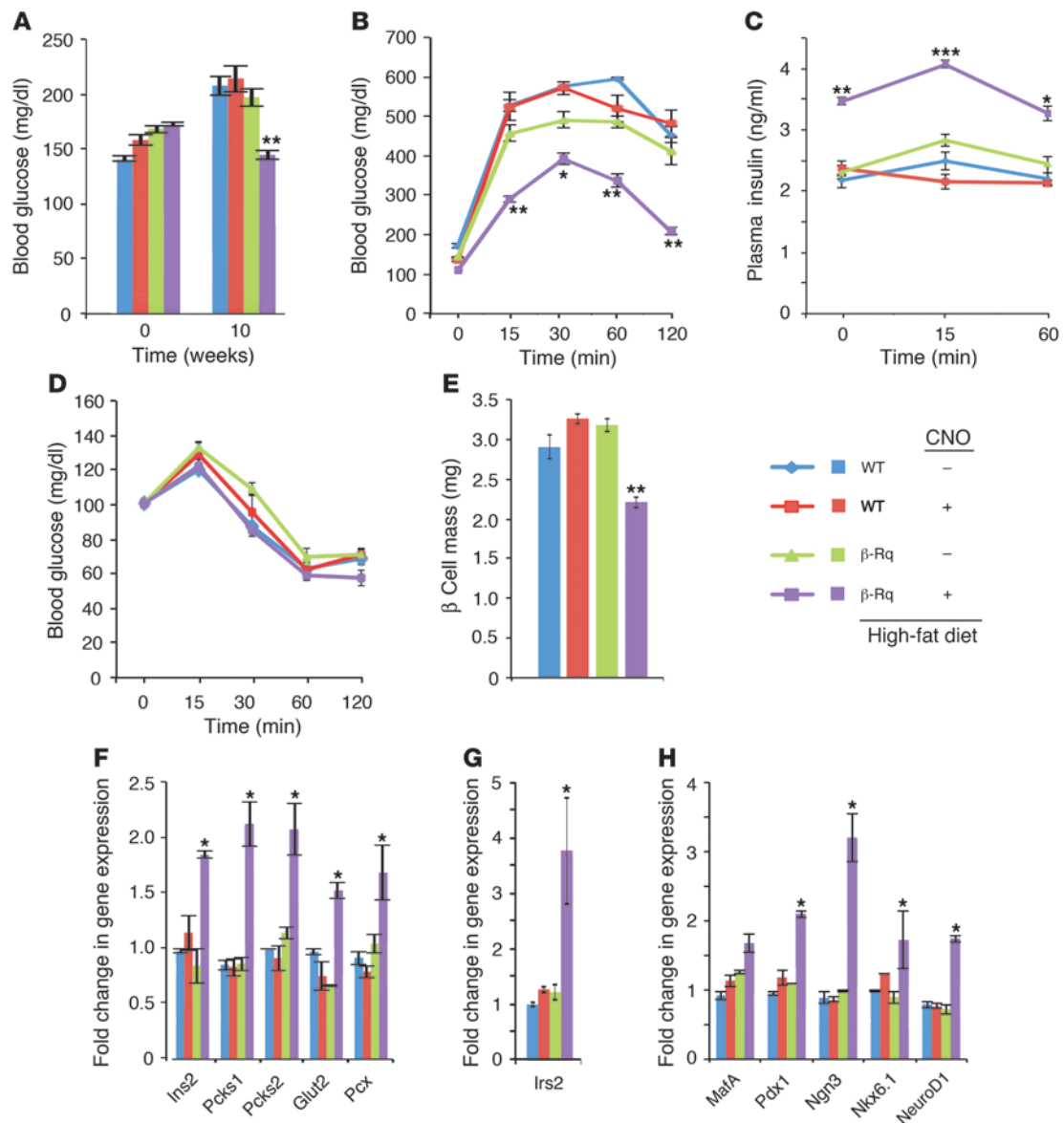
Figure 5
Beneficial metabolic effects of chronic activation of G_q signaling in β cells in a mouse model of diabetes. Where indicated, mice (8-week-old males) were injected with STZ for 5 consecutive days (50 mg/kg i.p. once per day). Mice received CNO via drinking water (0.25 mg/ml) for 10 weeks (CNO treatment was started 1 week prior to the first STZ injection). (**A–D**) Chronic CNO treatment of β-Rq mice prevents STZ-induced hyperglycemia (**A**; fasting blood glucose levels), hypoinsulinemia (**B**; fasting plasma insulin levels), glucose intolerance (**C**; IGTT, 2 mg/g glucose i.p.), and impaired glucose-induced (2 mg/g i.p) insulin release (**D**). (**E–I**) Chronic administration of CNO to STZ-treated β-Rq mice results in significantly increased pancreatic insulin content (**E**) and β cell mass (**F**) and enhances the expression levels of genes involved in insulin synthesis (**G**), Irs2 (**H**), and several important β cell transcription factors (**I**). Gene expression was studied by real-time qRT-PCR analysis of total islet RNA. Data were normalized relative to the expression of 18S rRNA and are presented as fold change in gene expression relative to non–CNO-treated WT mice. All experiments were performed at the end of the CNO treatment period. Data are expressed as mean ± SEM (**A–D**, $n = 6$ per group; **E–I**, $n = 3$ or 4 per group). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, as compared with the 3 control groups.

mice were 7 weeks old, they displayed metabolic impairments that were similar in magnitude to those observed with non–CNO-treated $IRS2^{-/-}$/β-Rq mice or $IRS2^{-/-}$ mice (Figure 4, A–D).

Gene expression studies demonstrated that IRS2 deficiency also led to a dramatic reduction in the expression of the same islet (β cell) genes that showed enhanced expression after activation of β cell G_q

signaling in β-Rq mice (compare Figure 4, E and F, with Figure 2, A–C). These genes included genes critical for insulin synthesis (Ins2 and Pcks1; Figure 4E) and many key β cell transcription factors (Figure 4F). Gene expression analysis of RNA prepared from islets of CNO-treated $IRS2^{-/-}$/β-Rq double-mutant mice indicated that chronic activation β cell G_q signaling failed to increase the low expres-

Figure 6
Chronic activation of G_q signaling in β cells prevents the metabolic deficits associated with the consumption of a HFD. All mice (males) were maintained on a HFD for 10 weeks. During this time, a subgroup of mice received CNO via drinking water (0.25 mg/ml). Mice started consuming the HFD when they were 4 weeks old. (**A** and **B**) Chronic administration of CNO to β-Rq mice prevents hyperglycemia (**A**; fed blood glucose levels) and glucose intolerance (**B**; IGTT, 2 mg/g glucose i.p.) observed with the 3 control groups. (**C**) Chronic treatment of β-Rq mice with CNO results in increased fasting plasma insulin levels (time 0) and enhanced glucose-induced (2 mg/g i.p.) insulin release, as compared with the 3 control groups. (**D**) All 4 groups of mice show a similar degree of insulin sensitivity (ITT). (**E**) CNO-treated β-Rq mice show a somewhat less pronounced increase in β cell mass, as compared with the 3 control groups. (**F**–**H**) Enhanced expression of key β cell genes in islets prepared from CNO-treated HFD β-Rq mice, including genes critical for insulin synthesis (**F**), *Irs2* (**G**), and several important β cell transcription factors (**H**). Islet gene expression was studied by real-time qRT-PCR using total islet RNA. Data were normalized relative to the expression of 18S rRNA and are presented as fold change in gene expression versus non–CNO-treated WT mice. Data are expressed as mean ± SEM (**A**–**D**, $n = 6$ per group; **E**–**H**, $n = 3$ per group). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, as compared with the 3 control groups.

sion levels of any of the genes caused by IRS2 deficiency (Figure 4, E and F). Taken together, these observations clearly demonstrate that the beneficial metabolic effects resulting from chronic activation of β cell G_q signaling require the presence (activity) of IRS2.

Chronic activation of β cell G_q signaling prevents diabetes in STZ-injected mice. To test the potential therapeutic relevance of these findings, we next examined whether chronic activation of β cell G_q

signaling in β-Rq mice might prove beneficial in a mouse model of diabetes. Specifically, we injected β-Rq mice and their WT littermates (8-week-old males) with a relatively low dose of STZ for 5 consecutive days (50 mg/kg i.p. daily; ref. 20). Previous work has shown that this treatment protocol does not lead to a complete destruction of β cells, but reduces β cell mass by approximately 70%–80% (20), thus mimicking the pronounced decrease in β cell
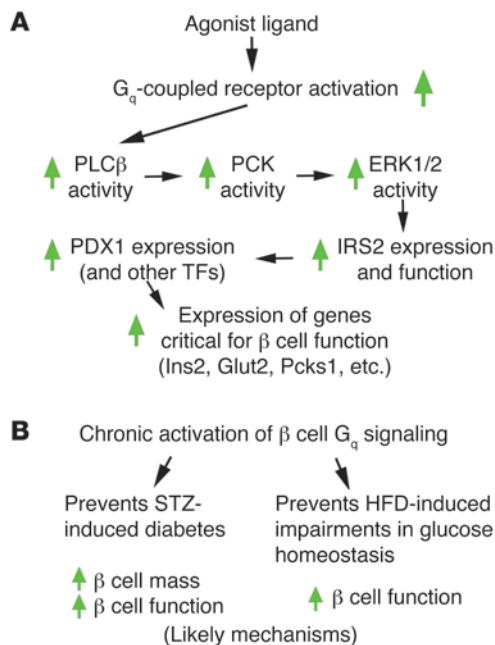
A

B

Figure 7
Biochemical (molecular) and physiological effects resulting from chronic activation of a β cell G_q-coupled receptor. (**A**) Depicted are some of the key signaling molecules involved in G_q-mediated activation of IRS2 expression and function in β cells. Enhanced IRS2 function is predicted to promote the expression of PDX1 and other important β cell transcription factors, resulting in the upregulation of many genes critical for β cell function. The scheme shown here is based on data described and discussed in this manuscript. (**B**) Highlighted are likely mechanisms by which chronic activation of G_q signaling in β cells prevents STZ-induced diabetes and HFD-induced impairments in glucose homeostasis (see text for details).

mass observed in patients suffering from T2D (21). A subgroup of transgenic and WT mice received CNO via drinking water (0.25 mg/ml) for 10 weeks (CNO treatment was started 1 week prior to the first STZ injection).

STZ-treated WT mice, independently of whether they were exposed to CNO or not, showed all the hallmarks characteristic of diabetes, including greatly elevated fasting blood glucose concentrations (Figure 5A), reduced plasma insulin levels (Figure 5B), glucose intolerance (Figure 5C), and pronounced deficits in glucose-induced insulin release (Figure 5D). Moreover, pancreata prepared from STZ-treated WT mice maintained on regular drinking water or on CNO water showed dramatic reductions in pancreatic insulin content (Figure 5E) and β cell mass (Figure 5F), as expected (20).

STZ-treated β-Rq mice consuming regular water showed metabolic deficits that were similar in magnitude to those observed with STZ-treated WT mice (Figure 5, A–F). In contrast, STZ-treated β-Rq mice maintained on CNO water showed fasting blood glucose and plasma insulin levels that were not significantly different from the corresponding levels observed with WT mice that had not been treated with STZ (Figure 5, A and B). Moreover, this group of mice showed largely normal glucose tolerance and glucose-induced insulin release (Figure 5, C and D), associated with significant increases in pancreatic insulin content and β cell mass (Figure 5, E and F). The increase in β cell mass observed with the STZ-treated β-Rq mice consuming CNO water was accompanied by significant increases in islet density and mean islet size, as compared with the 3 STZ-treated control groups (Supplemental Table 2 and Supplemental Figure 6). However, in contrast to mean islet size, pancreatic insulin content, β cell mass, and islet density did not reach the corresponding values observed with non–STZ-treated WT mice (Figure 5, E and F, and Supplemental Table 2). In an insulin tolerance test (ITT), all 4 groups of mice showed comparable decreases in blood glucose levels (Supplemental Figure 7).

To determine whether the metabolic phenotypes described above correlated with changes in islet (β cell) gene expression, we quantified the expression levels of various genes that are upregulated

after chronic activation of β cell G_q signaling in β-Rq mice. We found that islets prepared from STZ-treated β-Rq mice that had been maintained on CNO water showed selective increases (as compared with the 3 control groups) in the expression of genes involved in insulin synthesis (*Ins2*, *Pcks1*, and *Pcks2*; Figure 5G), *Irs2* (Figure 5H), and important β cell transcription factors (Figure 5I).

In conclusion, these findings clearly indicate that chronic activation of β cell G_q signaling can prevent diabetes in STZ-treated mice, most likely due to activation of cellular pathways that are critical for β cell function and proliferation.

Chronic activation of β cell G_q signaling prevents the metabolic deficits associated with the consumption of a HFD. An energy-rich HFD is known to trigger a number of metabolic impairments, including hyperglycemia and impaired glucose tolerance. To determine whether the severity of these metabolic deficits could be prevented by chronic activation of β cell G_q signaling, 4-week-old β-Rq mice and their WT littermates (males) were fed a HFD for 10 weeks. During this period, a subgroup of transgenic and WT mice received CNO via drinking water (0.25 mg/ml) for 10 weeks. When maintained on the HFD, the 4 groups of mice showed a similar degree of weight gain (~15–20 g at week 10).

The 3 control groups (WT and transgenic mice on regular water and WT mice on CNO water) showed significantly elevated blood glucose levels after consuming the HFD for 10 weeks (~200 mg/dl; mice had free access to food) (Figure 6A). In contrast, the blood glucose levels of β-Rq mice remained in a normal range (~150 mg/dl) under the same experimental conditions (Figure 6A). As expected, an i.p. glucose tolerance test (IGTT) demonstrated that the 3 control groups showed a high degree of glucose intolerance (Figure 6B). On the other hand, the CNO-treated β-Rq mice showed greatly improved (normal) glucose tolerance (Figure 6B). In agreement with the blood glucose measurements, fasting plasma insulin levels were significantly elevated (by ~50%–60%) in the β-Rq mice maintained on CNO water, as compared with the 3 control groups (Figure 6C). Moreover, following glucose administration (2 mg/g i.p.), the CNO-treated transgenic mice released significantly more insulin than the control animals (Figure 6C). In contrast, all 4 groups of mice showed comparable decreases in blood glucose levels in an ITT (Figure 6D).

As is commonly seen with mice maintained on a HFD, all 4 experimental control groups displayed significant increases in β cell mass, as compared with WT mice maintained on regular mouse chow (compare Figure 6E with Figure 1E). Somewhat surprisingly, the CNO-treated β-Rq mice showed a smaller increase in β cell mass than the 3 control groups, most likely due to other CNO effects that improve β cell function in HFD β-Rq mice (see the following paragraph).

Finally, we carried out qRT-PCR studies to determine the expression levels of key β cell genes using RNA prepared from pancreatic islets derived from the 4 experimental HFD groups. In general, we observed changes in gene expression patterns that were very similar to those found with mice maintained on regular chow or with STZ-treated mice. Specifically, we found that the CNO-treated β-Rq mice showed selective increases in the expression of islet genes involved in insulin synthesis (*Ins2*, *Pcks1*, and *Pcks2*), *Glut2*, *Pcx* (Figure 6F), *Irs2* (Figure 6G), and key β cell transcription factors (Figure 6H).

Discussion

In this study, we analyzed transgenic mice that express a G_q-coupled designer GPCR (Rq; ref. 10) selectively in their pancreatic β cells (β-Rq mice). Importantly, the Rq receptor is not recognized by endogenous ligands, but can be selectively activated by an exogenously administered drug, CNO, a pharmacologically inert compound (11).

Chronic CNO treatment of β-Rq mice maintained on regular mouse chow resulted in a robust increase in β cell mass, in agreement with a previous report (10). In the present study, we made the observation that chronic activation of β cell G_q signaling led to enhanced β cell proliferation, but had no significant effect on the rate of β cell apoptosis (Figure 1, F and G). This finding supports the concept that the increase in β cell mass triggered by chronically enhanced G_q signaling is caused primarily by an enhanced rate of β cell replication.

We also observed that chronic activation of β cell G_q signaling in β-Rq mice led to a significant increase in pancreatic insulin content (Figure 1C) and enhanced expression of the genes coding for preproinsulin (*Ins2*) and proprotein convertases 1 and 2 (*Pcks1* and *Pcks2*, respectively; Figure 2A). Taken together, these data indicate that persistent activation of β cell G_q signaling enhances pancreatic insulin content by promoting β cell proliferation and by simulating transcriptional processes leading to increased insulin synthesis.

Strikingly, gene expression studies using RNA from isolated pancreatic islets indicated that chronic activation of β cell G_q signaling triggered significant increases in the gene expression levels of several key transcription factors that determine β cell differentiation and/or are critical for maintaining normal β cell function and β cell mass, including Pdx1, MafA, Ngn3, NeuroD1, and Nkx6.1 (refs. 22–26 and Figure 2C). Pdx1 is considered to be the key transcription factor involved in β cell differentiation and replication and the maintenance of proper β cell function (22–25). Pdx1 acts in concert with MafA and NeuroD1 to strongly activate the insulin promoter and contributes to glucose-responsive insulin gene transcription (27, 28). Moreover, Pdx1 stimulates the expression of many functionally critical β cell genes, including *Ins2*, *Pcks1*, *Pcks2*, and *Glut2* (29, 30). These observations strongly support the concept that the increased expression of *Ins2*, *Pcks1*, *Pcks2*, *Glut2*, and other important β cell genes associated with chronic activation of β cell G_q signaling is most likely caused by enhanced expression of Pdx1 and, probably, other β cell transcription factors.

In this context, an important question is how persistent stimulation of β cell G_q signaling promotes the expression of *Pdx1* and various other important β cell transcription factors. Interestingly, gene expression profiling of RNA from isolated pancreatic islets demonstrated that chronic activation of β cell G_q signaling triggered a pronounced increase in the expression of *Irs2* (Figure 2B). This effect was observed with mice consuming regular chow or

a HFD as well as with STZ-injected mice. IRS2 represents a key intermediate in the signaling pathways activated by insulin and IGF-1 and is known to play a critical role in maintaining normal β cell function (13, 16–19).

Previous work has demonstrated that hyperinsulinemia leads to reduced *Irs2* expression levels in cultured cells and mouse peripheral tissues (13, 31). Since chronic stimulation of β cell G_q signaling was generally associated with increased plasma insulin levels (hyperinsulinemia), it is unlikely that the G_q-mediated increases in *Irs2* gene expression are simply the result of altered plasma insulin levels.

Western blotting studies confirmed that the prominent increase in *Irs2* transcript levels found with islets derived from CNO-treated β-Rq mice also resulted in increased IRS2 protein levels (Figure 2, D and E). In addition, we found that the expression levels of the phosphorylated, signaling-competent forms of Akt and ERK1/2 were also enhanced in islets derived from β-Rq mice chronically treated with CNO (Figure 2, D and E). Akt and ERK1/2 are 2 key kinases that mediate many of the important cellular functions of IRS2, including the stimulatory effects of IRS2 on β cell replication (13). Taken together, our findings clearly indicate that chronic activation of β cell G_q signaling promotes signaling through the IRS2-Akt-ERK1/2 cascade. Given the importance of this pathway for maintaining normal β cell function (13, 32, 33), it is likely that the beneficial metabolic effects caused by persistent stimulation of β cell G_q signaling are mediated, at least in part, by IRS2-dependent activation of this signaling pathway.

To gain insight into the mechanisms that link enhanced β cell G_q signaling to increased *Irs2* expression, we carried out additional studies with rat INS1-M3 insulinoma cells, which express moderate levels of the M_3 muscarinic receptor, a prototypic G_q-coupled receptor (15). We found that M_3 receptor–mediated increases in *Irs2* expression could be blocked by pharmacological inhibitors of PLC, PKC, and MEK1/2 and siRNA-mediated knockdown of ERK1/2 (Figure 3, D and E). We obtained similar findings when we studied the ability of CNO to stimulate *Irs2* expression and ERK1/2 phosphorylation in isolated islets prepared from β-Rq mice (Figure 3, F and G). These data strongly support the concept that the stimulatory effect on β cell *Irs2* expression following activation of a G_q-coupled receptor requires stimulation of the PLCβ/PKC pathway, which ultimately leads to enhanced *Irs2* promoter activity in an ERK1/2-dependent fashion (Figure 7A). Interestingly, a recent study (34) demonstrated that ERK activation substantially enhances the activity of the *Irs2* promoter by stimulating the binding of the SP1 and NFI transcription factors to a short promoter region.

In this context, it should be noted that previous studies have shown that stimulation of β cell G_s, a G protein mediating increases in intracellular cAMP levels via the activation of adenylyl cyclase, can also enhance the activity of the *Irs2* promoter in β cells. For example, treatment of MIN6 insulinoma cells with exendin-4, a drug that selectively activates the G_s-coupled GLP-1 receptor, leads to enhanced *Irs2* promoter activity involving cAMP/PKA-activated binding of CREB to the *Irs2* promoter (35). Several lines of evidence suggest that this action contributes to the beneficial metabolic effects of GLP1 receptor agonists on β cell function and growth (7, 35, 36). Since agonist activation of the Rq receptor has no effect on intracellular cAMP levels (ref. 10 and also see Figure 3A), the outcome of the present study supports the existence of a novel β cell pathway involving G_q-mediated activation of ERK1/2 through which IRS2 expression and signaling can be enhanced in a GPCR-dependent fashion.

To further explore the hypothesis that the beneficial metabolic effects on β cell function caused by chronic activation of β cell G_q signaling require the presence of IRS2, we generated and analyzed IRS2-deficient mice ($IRS2^{-/-}$ mice; ref. 16) that expressed the Rq receptor in a β cell–selective fashion ($IRS2^{-/-}/β$-Rq double-mutant mice). Importantly, chronic CNO treatment of $IRS2^{-/-}/β$-Rq double-mutant mice had no significant effect on the severity of the diabetes phenotype and the profound deficits in islet gene expression associated with IRS2 deficiency (Figure 4, A–F). These data provide additional support for the concept that IRS2 plays a central role in mediating the beneficial effects on β cell function triggered by chronic activation of β cell G_q signaling in vivo.

Studies with *Irs2* mutant mice have shown that IRS2 is able to promote *Pdx1* expression (16, 18). It is therefore likely that the increase in *Pdx1* expression resulting from chronic activation of β cell G_q signaling is due, at least in part, to increased IRS2 expression and activity (Figure 7A).

To examine the potential clinical relevance of persistently stimulating β cell G_q signaling, we induced diabetes in β-Rq mice via a series of STZ injections that resulted in a severe reduction (but not a complete loss) in β cell mass (Figure 5). It should be noted in this context that human autopsy studies demonstrated that obese patients with T2D displayed an at least 50% reduction in β cell mass (21). Interestingly, STZ-injected β-Rq mice that had been chronically treated with CNO displayed normal blood glucose and plasma insulin levels, glucose tolerance, and glucose-induced insulin release, similar to non–STZ-treated WT mice (Figure 5, A–D). These striking metabolic improvements were most likely caused by the pronounced increases in pancreatic insulin content and β cell mass resulting from CNO treatment of STZ-injected β-Rq mice (Figure 5, E and F). Chronic activation of β cell G_q signaling in STZ-injected β-Rq mice also greatly stimulated the expression levels of the same set of genes as observed with CNO-treated β-Rq animals not injected with STZ (see above; Figure 5, G–I). As discussed elsewhere, it is likely that these changes at the transcriptional level trigger the cellular events that promote improved β cell function and enhanced β cell mass in this mouse model of diabetes (Figure 7, A and B). Despite the findings outlined above, the precise mechanisms through which chronic activation of β cell G_q signaling prevents STZ-induced diabetes remain to be elucidated. In the present study, we started to administer CNO prior to STZ treatment. In future experiments, we are planning to initiate CNO treatment at a later point, after the STZ-induced loss of β cell mass has already occurred. This experimental strategy should reveal whether or not chronic activation of β cell G_q signaling is able to promote β cell replication in diabetic mice suffering from a severe reduction in β cell mass.

Finally, we maintained β-Rq mice on a HFD to induce obesity-associated metabolic deficits. Consumption of the HFD led to hyperglycemia, glucose intolerance, and impaired glucose-induced insulin release (Figure 6, A–C). Importantly, all these metabolic deficits could be prevented when β-Rq mice were chronically treated with CNO (Figure 6, A–C). Interestingly, we previously reported that acute treatment of HFD β-Rq mice with a single dose of CNO (1 mg/kg i.p.) also resulted in pronounced improvements in glucose tolerance and glucose-induced insulin release (10). Chronic CNO treatment of HFD β-Rq mice greatly stimulated the expression levels of the same β cell genes, including *Irs2* and several other genes critical for β cell function and maintenance, as observed with CNO-treated β-Rq mice consuming regular mouse chow (Figure 6, F–H). It is therefore likely that the resulting improvement in β cell function is responsible for the beneficial metabolic effects observed with CNO-treated HFD β-Rq mice.

As discussed above, chronic CNO treatment of STZ-injected β-Rq mice led to a robust increase in β cell mass. In contrast, when mice were maintained on a HFD, the CNO-treated β-Rq mice showed a smaller increase in β cell mass than the 3 control groups (non-treated β-Rq and WT mice and CNO-treated WT mice; Figure 6E). One possible explanation for this surprising finding is that CNO treatment of HFD β-Rq mice caused beneficial metabolic effects primarily by improving β cell function (see previous paragraph and Figure 7B). Clearly, additional studies are required to gain more detailed insight into the relationship between improved β cell function and changes in β cell mass in the different mouse models used in the present study.

Pancreatic β cells express various G_q-coupled receptors, including the M_3 muscarinic receptor, GPR40, GPR120, and different P2Y receptor subtypes (5). These GPCRs represent promising drug targets, particularly given the outcome of the present study. In agreement with this view, initial clinical studies have shown that TAK-875, an orally bioavailable GPR40 agonist, is able to lower blood glucose levels in diabetes patients (37). Although the receptors listed above are also expressed by other tissues and cell types, the possibility exists that activating ligands can be administered at doses that preferentially target β cells, perhaps due to higher receptor expression levels and/or greater receptor coupling efficiency to downstream signaling pathways in this cell type. Moreover, the development of so-called allosteric enhancers, which are able to sensitize specific GPCRs to activation by their endogenous agonists, may lead to novel antidiabetic drugs endowed with reduced side effects (38).

In conclusion, we delineated a novel β cell signaling pathway, activation of which has multiple beneficial metabolic effects in mice treated with STZ or maintained on a HFD. Given the need for novel classes of antidiabetic drugs, our findings should be of considerable clinical relevance.

Methods

Mice. The β-Rq transgenic mice were obtained and genotyped as described previously (10). These mutant mice were maintained on a pure C57BL/6NTac background. The generation of IRS2-deficient mice ($IRS2^{-/-}$ mice) has been reported previously (16). We crossed $IRS2^{-/-}$ mice with β-Rq transgenic mice to obtain $IRS2^{-/-}$ mice that expressed the Rq receptor selectively in pancreatic β cells ($IRS2^{-/-}/β$-Rq double-mutant mice).

Mouse maintenance and diet. Mice were fed ad libitum and kept on a 12-hour light/12-hour dark cycle. Unless stated otherwise, all experiments were carried out with male littermates that were 10 to 16 weeks old and maintained on a standard mouse chow (4% [w/w] fat content; Zeigler). In a subset of experiments, 4- to 5-week-old mice were put on a HFD (35.5% [w/w] fat content; # F3282, Bioserv) for at least 10 weeks.

STZ-induced diabetes. To generate a mouse model of diabetes, mice (8-week-old males) received daily injections of STZ (Sigma-Aldrich) for 5 consecutive days (50 mg/kg i.p.) following a protocol described by Maida et al. (20).

Chronic CNO administration. CNO was obtained from the NIH as part of the Rapid Access to Investigative Drug Program funded by the National Institute of Neurological Disorders and Stroke (NINDS). To chronically activate G_q signaling selectively in pancreatic β cells, β-Rq mice received daily injections of CNO (1 mg/kg i.p. per day) or consumed drinking water containing CNO at a concentration of 0.25 mg/ml (for up to 4 weeks).

Physiological studies. The i.p. IGTTs and ITTs were carried out in mice that had been fasted overnight for 10 to 12 hours (for details, see Supplemental Methods).

Real-time qRT-PCR studies. Total RNA was prepared from isolated mouse pancreatic islets (3 to 4 mice per group per experiment). Subsequently gene expression levels were determined via real-time qRT-PCR (for details, see Supplemental Methods, including Supplemental Table 3).

Determination of pancreatic insulin content. Total pancreatic insulin content was measured by using an acid-ethanol method, as described previously (39).

Morphometric analysis of pancreatic islets. Islet morphometric studies were performed using 10- to 16-week-old mice (3 or 4 mice per group) by employing standard techniques (for details, see Supplemental Methods).

Islet isolation. Pancreatic islets were hand picked from digested pancreatic tissue as described previously (39).

Western blotting studies. Western blotting studies were carried out with lysates from pancreatic islets or INS1-M3 cells using standard techniques (see Supplemental Methods and Supplemental Table 4 for details).

Studies with INS1-M3 insulinoma cells. The INS1-M3 insulinoma cell line, which expresses moderate levels of M_3 muscarinic receptors, was provided by Trevor Biden (Garvan Institute of Medical Research, Sydney, Australia). INS1-M3 cells were grown as described (14). To study M_3 receptor–mediated increases in *Irs2* expression, INS1-M3 cells were incubated in 6-well plates (~2×10^6 cells/well) for 5 hours at 37°C with the muscarinic agonist OXO-M (100 μM; incubation volume: 2 ml). The composition of the incubation medium has been described previously (14).

To explore the involvement of distinct kinases or signaling pathways in OXO-M–induced increases in *Irs2* expression, cells were treated with the following pharmacological inhibitors (in the presence of OXO-M): H-89 (PKA inhibitor; 10 μM), U73122 (PLC inhibitor; 10 μM), GF109203X (PKC inhibitor; 2.5 μM), and U0126 (MEK1/2 inhibitor; 10 μM).

For gene-silencing studies, INS1-M3 cells (~$1–1.5 \times 10^6$ cells) were electroporated with 100 nmoles of siRNA according to the manufacturer's instructions (Nucleofector II; Amaxa). siRNAs targeting rat ERK1 (NM 017347) and ERK2 (NM 053842) were purchased from Dharmacon Research (40). Scrambled control siRNA was obtained from Life Technologies (Ambion Silencer Negative Control #1, AM4611). Electroporated cells were grown in 6-well plates for ~48 hours and then treated with OXO-M to monitor M_3 receptor–mediated increases in *Irs2* expression, as described above. Cell viability was assessed by using a colorimetric assay (MTT kit; # 7H258, Sigma-Aldrich) according to the manufacturer's instructions.

Drug-induced increases in intracellular calcium levels were determined in 96-well plates using FLIPR technology (Molecular Devices) essentially as described (41).

For cAMP assays, INS1-M3 cells were plated into 384-well solid-bottom white plates (750 cells per well; CN:784075, Greiner Bio-One). Cells were incubated with increasing concentrations of CNO or forskolin in INS1-M3 medium containing 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX). Incubations were carried out for 70 minutes at 37°C. Subsequently, changes in intracellular cAMP levels were determined by homogeneous time-resolved fluorescence using the Cisbio cAMP Dynamic 2 kit (CN:62AM4PEB; Cisbio Bioassays) according to the manufacturer's instructions.

CNO-induced Irs2 expression and ERK1/2 phosphorylation in isolated β-Rq islets. Pancreatic islets were freshly prepared from β-Rq mice (10-week-old females) and cultured overnight in 12-well plates (40 islets per well) in a 5% CO_2 incubator at 37°C in RPMI 1640 cell culture medium (Invitrogen) supplemented with 5.5 mM glucose, 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. One hour prior to the addition of CNO, the culture medium was replaced with serum-free assay medium (RPMI 1640 medium containing 3.3 mM glucose, 120 mM NaCl, 5 mM KCl, 1.1 mM $MgCl_2$, 2.6 mM $CaCl_2$, and 25 mM $NaHCO_3$). Pharmacological inhibitors were added 30 minutes prior to the addition of CNO. To monitor CNO-mediated increases in *Irs2* expression, islets were incubated with CNO (1 μM) for 3 hours. For ERK1/2 phosphorylation studies, islets were incubated with CNO (1 μM) for 10 minutes.

Statistics. Data are expressed as means ± SEM for the indicated number of observations. Statistical significance between groups was determined using 2-tailed Student's *t* test or 1-way ANOVA followed by appropriate post hoc tests. $P < 0.05$ was considered significant.

Study approval. All animal studies were approved by the National Institute of Diabetes and Digestive and Kidney Diseases/NIH Animal Care and Use Committee.

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