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Sustained NF-κB Activation and Inhibition in β-Cells Have Minimal Effects on Function and Islet Transplant Outcomes

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Abstract

The activation of the transcription factor NF-κB leads to changes in expression of many genes in pancreatic β-cells. However, the role of NF-κB activation in islet transplantation has not been fully elucidated. The aim of the present study was to investigate whether the state of NF-κB activation would influence the outcome of islet transplantation. Transgenic mice expressing a dominant active IKKβ (constitutively active) or a non-degradable form of IκBα (constitutive inhibition) under control of the rat insulin promoter were generated. Islets from these mice were transplanted into streptozotocin diabetic mice in suboptimal numbers. Further, the effects of salicylate (an inhibitor of NF-κB) treatment of normal islets prior to transplantation, and the effects of salicylate administration to mice prior to and after islet implantation were evaluated. Transplantation outcomes were not affected using islets expressing a non-degradable form of IκBα when compared to wild type controls. However, the transplantation outcomes using islets isolated from mice expressing a constitutively active mutant of NF-κB were marginally worse, although no aberrations of islet function *in vitro* could be detected. Salicylate treatment of normal islets or mice had no effect on transplantation outcome. The current study draws attention to the complexities of NF-κB in pancreatic beta cells by suggesting that they can adapt with normal or near normal function to both chronic activation and inhibition of this important transcription factor.

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Introduction

Inflammatory cytokines such as INF-γ, TNF-α and IL-1 have been implicated in the autoimmune destruction of pancreatic β-cells in type 1 diabetes [1]. Since NF-κB is both activated by these cytokines, and drives their expression, considerable interest has been focused on NF-κB in β-cells [2,3]. But the situation is complex because NF-κB may increase the expression of both proapoptotic and antiapoptotic genes, and patterns of gene expression may vary depending on context and cell type. In β-cells, cytokine-induced activation of NF-κB has been associated with increased expression of inflammatory proteins such as iNOS and COX-2, and nitric oxide (the product of iNOS) has been implicated in IL-1β-induced β-cell death [4,5]. NF-κB activation has also been associated with the enhanced expression of proapoptotic and protective genes [6–8]. *In vitro* studies have shown that the inhibition of NF-κB can protect beta cells against cytokine-induced death [9–11]. However, others have suggested that NF-κB activation could play a protective role preventing TNF-induced β-cell apoptosis [12]. Indeed, it has been suggested that NF-κB may play a biphasic role in cytokine-induced β-cell death, by initially protecting the β-cells before leading to apoptosis [13]. It has also been recently suggested that NF-κB may act as an antiapoptotic factor in normoxic conditions but act as an apoptotic factor in hypoxic conditions [14]. Studies have shown that genetically modified mice with disrupted NF-κB may be resistant to β-cell toxins, such as multiple low-dose streptozotocin injections [15,16]. In transplantation settings it has been suggested that acute inhibition of NF-κB can improve islet transplantation outcome [14,17–21].

Transplantation of islets is an important breakthrough in the treatment of Type 1 diabetes [22]. It can reverse hyperglycaemia in humans [23], but long-term success is limited [24], indicating a failure to maintain islet mass. Because NF-κB is a potentially useful therapeutic target and seems to be involved in β-cell destruction in models of diabetes, we sought to determine if the state of NF-κB activation would influence the outcome of islet transplantation.

The *in vivo* activity of NF-κB is tightly regulated by an inhibitory protein, IκBα [25] and an activating kinase, IKKβ [26]. Once proinflammatory stimuli have activated IKKβ, it phosphorylates IκBα, which is targeted for ubiquitination and proteasomal

degradation. The liberated NF-κB translocates to the cell nucleus and drives transcription. To study the regulation of β-cell function by NF-κB, transgenic mice expressing a dominant active IKKβ to activate NF-κB (βIKK) or a non-degradable form of IκBα to prevent NF-κB activation (βISR) under control of the rat insulin promoter (RIP) were generated. In addition to these genetic approaches, NF-κB activity was modulated both *in vivo* and *in vitro* using salicylates [27]. Salicylate inhibits NF-κB, and forms of salicylate including salsalate are being investigated as a potential new therapeutic modality in patients with diabetes [27–30].

Methods

Ethics

The Joslin Animal Care Committee approved all animal experiments.

Establishing transgenic mice

βIKK and βISR mice were created as described previously for both skeletal muscle (MIKK, MISR) and liver (LISR, LIKK) specific expression of dominant active IKKβ (S177/181E) and a non-degradable form of IκBα (S32/36A; super repressor), respectively [31,32]. To produce βIKK and βISR mice, IKKβ (S177/181E) and IκBα (S32/36A), respectively, were expressed selectively in β-cells using the rat insulin 2 promoter. N-terminal FLAG or His tag sequences were included in exon 2 of a β-globin splicing cassette (Figure 1). The DNA fragments were released by Pme1 enzyme digestion and microinjected into the pronuclei of C57BL/6 oocytes, which were then implanted into pseudopregnant female mice in the Joslin Transgenic Mouse Facility. Three founders for βIKK and two for βISR were identified by tail DNA genotyping (Figure 2). These mice were created using C57BL/6 mice, and are thus 100% C57BL/6 background.

Visualization of NF-κB in dispersed cells

NF-κB activation was assessed by immunocytochemistry. Cells in isolated islets from mice 8–12 weeks of age were dispersed using 1 mg/ml trypsin in Ca^{2+} and Mg^{2+} free Hanks' solution at 37°C for 3 min as described previously [33] washed in PBS and centrifuged onto glass slides. The cellular localization of NF-κB was examined by immunofluoresence as described previously [34] using rabbit anti nuclear p50 (1:100 dilution, Santa Cruz, Santa Cruz, CA) and guinea-pig anti-human insulin (1:100 dilution, DakoCytomation, Carpinteria, CA). Secondary antibodies Alexa-Fluor 488 goat anti guinea-pig IgG (Molecular Probes, Eugene, OR) and CY3 conjugated donkey anti-rabbit (Jackson Immunoresearch Laboratories, West Grove, PA) were used as a 1:200 dilution and nuclei were detected using DAPI (Sigma) Slides were visualized using a Nikon Eclipse 90I.

Visualization of NF-κB in formalin fixed pancreases and grafts

Pancreases or grafts were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained for NF-κB using a p65 antibody (Abcam, Cambridge, MA). Briefly, slides were hydrated and triton x was applied to permeabilise the membranes. Antigen retrieval was carried out using citric acid and a pressure cooker. Slides were blocked by avidin and biotin prior to H_2O_2 quenching. Donkey serum (1:50) was added for 30 min prior to the antibody, which was added overnight at 4°C. Tyramide amplification was carried out prior to the addition of the secondary antibody (Donkey anti-rabbit Alexoflour, 1:400) for 1 hour.

Glucose tolerance tests (GTTs) with βIKK and βISR mice

GTTs were performed on 8–12 wk old βIKK and βISR mice, as well as age and weight matched wild-type siblings. After an overnight fast, mice were injected i.p. with 2 g/kg glucose. Blood glucose levels were measured prior to the injection and at 15, 30, 60, 90 and 120 min using a glucose meter (Accu-Check; Boehringer-Mannheim Biochemicals, Indianapolis, IN) with blood obtained from a snipped tail.

Mice receiving islet transplants

Male C57BL/6AF1 mice (Jackson Laboratories, Bar Harbor, ME) age 6-10 weeks were used as donors and recipients of islet grafts. Transgenic βIKK or βISR mice were also used as transplant donors. Recipient animals were made diabetic with a single i.p. injection of streptozotocin (Sigma, St Louis, MO) 180 mg/kg body wt, freshly dissolved in citrate buffer (pH = 4.5). Only those mice with blood glucose levels greater than 20 mmol/l were used as recipients.

Islet Isolation

Islets were isolated using collagenase digestion followed by separation with a density gradient as previously described in detail [35]. After isolation, islets were handpicked and transplanted immediately, or cultured for 72 h in RPMI 1640+10% FCS in the absence or presence of 2 mmol/l salicylate.

Islet transplantation

Animals were anaesthetised using 0.02 ml/g BW Avertin [2.5% (vol/vol) solution of 10 g 97% 2.2.2-tribromoethanol (Sigma) in 10 ml 2-methyl-2-butanol]. The left kidney was exposed through a lumbar incision and the kidney capsule was incised. Using a Hamilton syringe (Fisher, Pittsburg, PA) and polyethylene tubing (Cole Parmer, Vernon Hills, IL), islets were placed under the kidney capsule as previously described [36]. A suboptimal number of 150 islets were used to assess the efficacy of the transplants.

Salicylate treatment of mice receiving islet transplants

Recipient male C57BL/6AF1 mice were injected with 180 mg/kg streptozotocin eight days before transplantation. Three days before transplantation, diabetic animals having non-fasting blood glucose levels >20 mmol/l were randomly assigned to receive water with or without salicylate. Mice were housed separately and consumption of water was calculated by weighing the water bottles every 24 h. The concentration of the salicylate was calculated based on the previous 24 h consumption of water, giving each animal approximately 30 mg salicylate/day. Salicylate administration was begun 3 days before transplantation. Mice were transplanted with 150 freshly isolated islets under the left kidney capsule. Blood glucose levels and water consumption were monitored daily. The concentration of salicylate in the water was modified as blood glucose levels fell and the mice were drinking less water. Salicylate administration was continued until the end of the study (28 days), irrespective of whether or not the animals were cured.

Salicylate administration to cultured islets

Islets were cultured in RPMI 1640 and 10% foetal calf serum in petri dishes in groups of approximately 200 islets for 72 h as described above. The media contained a salicylate concentration of 2 mmol/l, which was changed every 24 h. For transplantation, 150 islets were handpicked and transplanted as described above. Blood glucose levels and weights were monitored weekly and i.p. GTTs were carried out 10 wk after transplantation in cured mice.

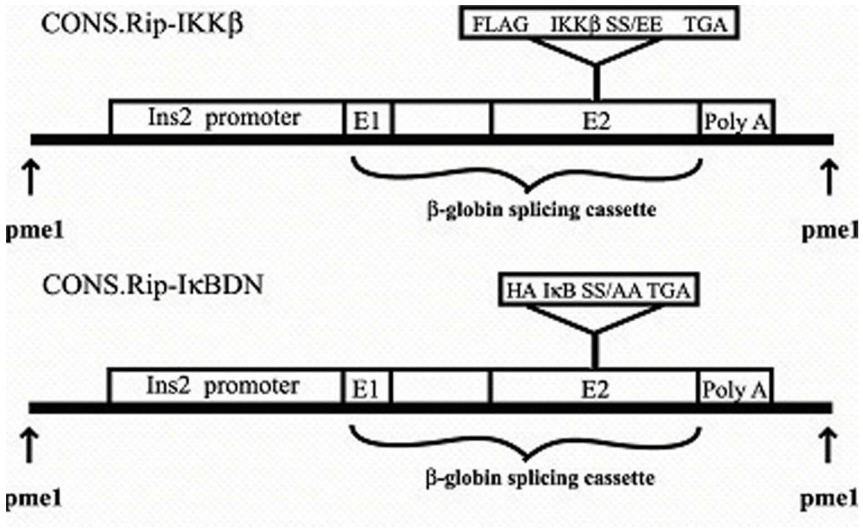

Figure 1. Constructs used to create βIKK and βISR mice. Dominant active IKKβ (S177/181E) or a non-degradable form of IkBα (S32/36A; super repressor) were expressed selectively in beta cells using the rat insulin 2 promoter to produce the βIKK and βISR mice, respectively. N-terminal FLAG or His tag sequences were included in exon 2 of a β-globin splicing cassette
doi:10.1371/journal.pone.0077452.g001

Insulin secretion and content of islets

To study insulin secretion *in vitro*, triplicate groups of ten islets were placed in glass vials containing 250 µl Krebs-Ringer bicarbonate buffer supplemented with 10 mmol/l HEPES (Sigma-Aldrich), hereafter referred to as KRBH buffer. In addition, the KRBH was supplemented with 2 mg/ml bovine serum albumin (fraction V; MP Biomedicals Inc, Aurora, OH, USA) and 1.7 mmol/l glucose or 16.7 mmol/l glucose, for the first and second hour of incubation, respectively. Islets were either cultured in the presence or absence of 2 mmol/l salicylate for 72 h and then islets from each group were then split into two groups with or without 2 mmol/l salicylate. After the secretion experiments, the islets were pooled into groups of 30 and insulin was extracted overnight at 4°C in acid ethanol to determine insulin content. Insulin concentrations were determined by insulin ELISA (Mercodia Rat Insulin ELISA; Mercodia AB, Uppsala, Sweden).

Glucose oxidation measurements

Islet glucose oxidation rates were determined with a previously described method [37]. Triplicate groups of ten islets were incubated in glass vials containing 100 µl Krebs-Ringer bicarbonate buffer supplemented with 10 mmol/l HEPES (Sigma-Aldrich), hereafter referred to as KRBH buffer. The KRBH was supplemented with D-[U-^{14}C] glucose (0.3 mCi/mmol/l, Amersham, UK) and non-radioactive D-glucose to give final glucose concentrations of 1.7 and 16.7 mmol/l. The $^{14}CO_2$ formed by cell metabolism was entrapped in hyamine and measured by liquid scintillation counting.

Viability of islets

The viability of islets was measured using propidium iodide and Hoechst staining. Five isolated islets were analysed from each mouse with an average of 361±20 cells counted in each islet and an average of 1809±152 cells counted per animal. Islets from five βIKK and five wild-type mice were analysed in a blind fashion. Mice between 16 and 24 wk of age were matched with siblings. A mixture of 2 mg/ml propidium iodide (Sigma) and 0.5 mg/ml bisbenzimide (Hoechst 33258, Sigma) was added to the islets, which were incubated at 37°C for 15 min, then washed with PBS and put on a coverslip. Images were taken using a Zeiss Axiocam camera on a fluorescent microscope (Leica, Leitz DMRB) with a UV-2B filter and Openlab 3.0.4 software. The total number of cells living (stained blue) and dead cells (stained red) were counted using ImageJ (rsb.info.nih.gov/ij/).

In vitro evaluation of βIKK islets

In the βIKK islets, insulin release measurements, glucose oxidation measurements and viability studies were performed in fresh islets. In addition, βIKK islets were cultured in RPMI 1640 media supplemented with 10% FCS. Islets were cultured either in control conditions (11.1 mmol/l glucose) or with a high glucose concentration (33 mmol/l), or in the presence of IL-1β (2.5 U/ml) for a period of 48 h. After this period, insulin release and content, and glucose oxidation were measured.

Real time PCR for insulin mRNA was carried out using a method previously described in detail [38], using the following primers:

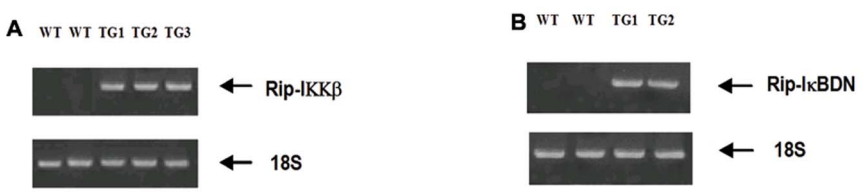

Figure 2. Genotyping of founder βIKK (A) and βISR (B) mice.
doi:10.1371/journal.pone.0077452.g002

insulin forward: 5′-ACAGCACCTTTGTGGTCC
insulin reverse: 5′-GGACTCAGTTGCAGTAGTTC
β-actin forward:5′-GCCCTGGCTCCTAGCACC
β-actin reverse: 5′-CCACCAATCCACACAGAGTACTTG

Statistical analysis

Values are expressed as mean±SEM. When two groups were compared, unpaired two-tailed Student's t-test was used. When more than two groups were compared an analysis of variance (ANOVA) was used. Repeated measurement ANOVA (RM ANOVA) was used when the same groups were tested at different time-points. Two way ANOVA was used when more than one variable was being considered. If the ANOVA was significant, the Student-Newman-Keuls (SNK) post hoc test was performed.

For all comparisons, p values of less than 0.05 were considered statistically significant. All statistics were carried out using Sigmastat 3.1 (Systat Software, Erkrath, Germany).

Results

Characterization of NF-κB in βIKK and βISR beta cells

Using an antibody specific for nuclear p50, the activation state of NF-κB in β-cells of βIKK and βISR mice was evaluated by immunofluorescence. NF-κB is not present in the nucleus of insulin containing cells isolated from wild-type mice, however, following a 30 min treatment with 10 U/ml IL-1β, NF-κB is nuclear localized in ~50% of insulin containing cells (pink, Figure 3). In islet cells isolated from βISR mice, IL-1β failed to stimulate NF-κB nuclear localization in β-cells, but did induce nuclear localization of NF-κB in some non-β-cells. NF-κB is constitutively nuclear in β-cells obtained from βIKK mice. These findings confirm the predicted activation state of NF-κB in these transgenic mice.

Glucose tolerance of βIKK and βISR mice

There were no differences in i.p. GTTs, with both female and male βISR mice showing similar glucose clearance as wild-type weight-matched gender-matched littermates at 12–16 weeks of age (Figure 4, female mice). Lean βIKK male and female mice also show similar glucose tolerance as their wild-type weight-matched gender-matched littermates at 10–14 weeks of age (Figure 4, female mice; males not shown).

Salicylate treatment of mice receiving islet transplants

The consumption of water during the 24 h time period prior to islet transplantation was similar in both groups of mice (18.7±1.1 ml/mouse/24 h in control mice and 18.5±2.1 in mice receiving water with salicylate). When a mouse became normoglycaemic (non-fasting blood glucose <11.1 mmol/l), its water consumption dropped to 3–5 ml per 24 h. Three of 11 salicylate-treated and 8 of 14 control mice were cured during the course of the study. The blood glucose concentrations in mice administered salicylate were not significantly different from control mice (Figure 5, 2 way RM ANOVA; $p = 0.153$ for salicylate treatment, $p<0.001$ for time passed after transplantation).

Transplantation results from islets cultured with salicylate

When islets were cultured for 72 h with salicylate and then transplanted, the blood glucose levels of the recipient mice tended to be lower than in mice with control islets (Figure 5, two way RM ANOVA; $p = 0.072$ for salicylate treatment, $p = 0.401$ for time). Twelve weeks after transplantation, diabetes was cured in 33% (4 of 12) of control mice (non-fasting blood glucose <11.1 mmol/l). By contrast, 64% (9 of 14) mice that had received salicylate-cultured islets had been cured. After culturing the islets with salicylate for 72 h, insulin release rates at 16.7 mmol/l glucose were lower than in islets cultured identically but without salicylate (Figure 6, $p = 0.035$, RM ANOVA with Student-Newman-Keuls (SNK) post hoc test). Islets exposed to salicylate only during the insulin release experiment did not have different insulin release rates than the control islets ($p = 0.161$, RM ANOVA with Student-Newman-Keuls (SNK) post hoc test).

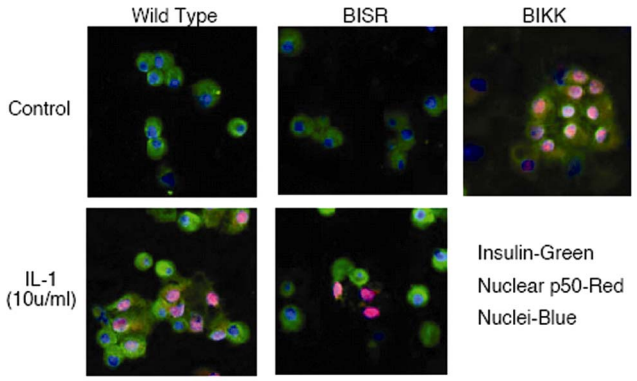

Figure 3. Translocation of NF-κB to the nucleus in islet cells from βIKK and βISR 8–12 week old mice in the presence or absence of 10 U/ml IL-1, using immunohistochemical detection of the NF-κB subunit p50, by an antibody specific for nuclear localized p50.
doi:10.1371/journal.pone.0077452.g003

Transplantation of βISR and βIKK islets

Transplantation of βISR islets resulted in similar blood glucose levels as transplantation of islets from wild-type littermates (Figure 7). After transplantation of βIKK islets, the recipient mice had higher blood glucose levels at 14 and 56 days compared with mice that received islets from wild-type littermates (Figure 7). At day 56, 64% of mice transplanted with βIKK islets remained overtly diabetic (blood glucose >20 mmol/l) as compared to 23% of mice transplanted with wild-type islets. In mice transplanted with βISR islets, 37% were overtly diabetic at day 56 compared to 27% of mice transplanted with wild-type islets. When grafts were studied for the presence of NF-κB using immunohistochemistry, it was evident that the βIKK grafts showed a higher activation of NF-κB (as demonstrated by its nuclear localization) whereas wild-type grafts showed little nuclear NF-κB staining (Figure 8).

In vitro function of βIKK islets

To better understand why the βIKK islets were less effective in reversing hyperglycaemia, we studied their function in vitro. There were no differences in insulin release rates at 16.7 mmol/l glucose, comparing freshly isolated βIKK vs. wild-type islets (16.9±3.6 vs. 16.6±3 ng insulin/10islets/h, respectively; $p = 0.72$, t-test). Moreover, glucose oxidation rates were similar for freshly isolated βIKK and wild-type islets cultured either at 1.7 mmol/l glucose (25±7 and 29±4 pmol/10 islets/90 min, respectively; $p = 0.74$, t-test) or 16.7 mmol/l glucose (208±27 and 173±43 pmol/10islets/90 min, respectively; $p = 0.51$, t-test). Insulin mRNA expression was similar as well, as measured by real time PCR, in freshly isolated βIKK islets compared with wild-type islets (705±49 vs 683±72 arbitrary units when normalized to actin, respectively; t-test, $p = 0.81$, $n = 5$). However, there was a modest but significant decrease in the viability of βIKK islets directly after isolation compared with wild-type islets (89.8±0.8 vs 93.6±0.8%, $p = 0.0013$, t-test, $n = 5$).

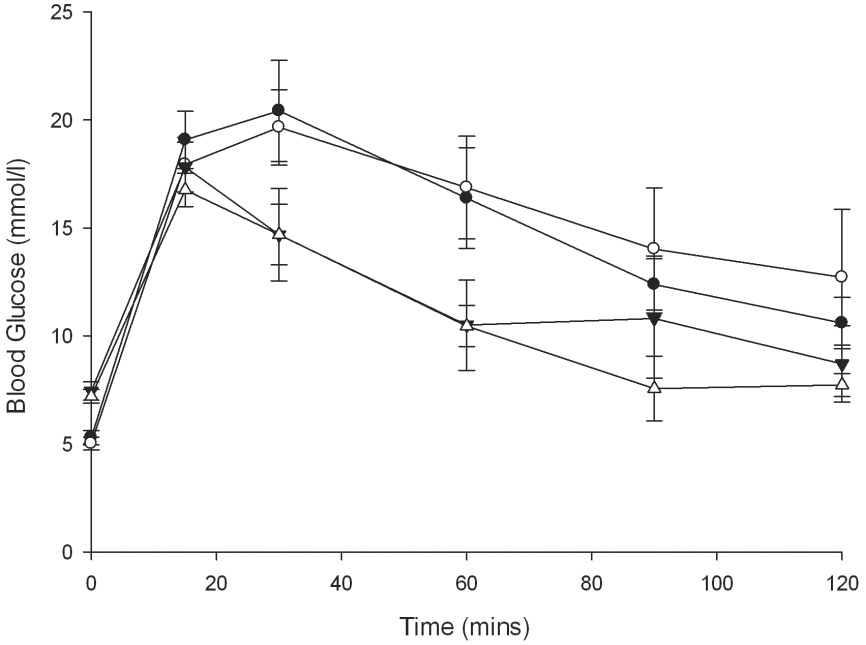

Figure 4. Intraperitoneal glucose tolerance tests (2 g/kg) conducted in female βISR (filled circle) vs. wt littermate control (open circle) mice (n = 6–7) and in female βIKK (filled triangle) vs. wt littermate control (open triangle) mice (n = 4–5).
doi:10.1371/journal.pone.0077452.g004

After 48 h culture periods, GSIS (stimulation index of glucose induced insulin secretion) tended to be lower for βIKK islets than control islets, but this did not reach significance (Table 1). Culture of islets for 48 h in the presence of 2.5 U/ml IL-1β reduced GSIS to similar levels in βIKK and wild-type islets (Table 1). In addition, culture of the islets in 33 mmol/l glucose for 48 h prior to the insulin release experiment also had similar effects in both βIKK and wild-type islets.

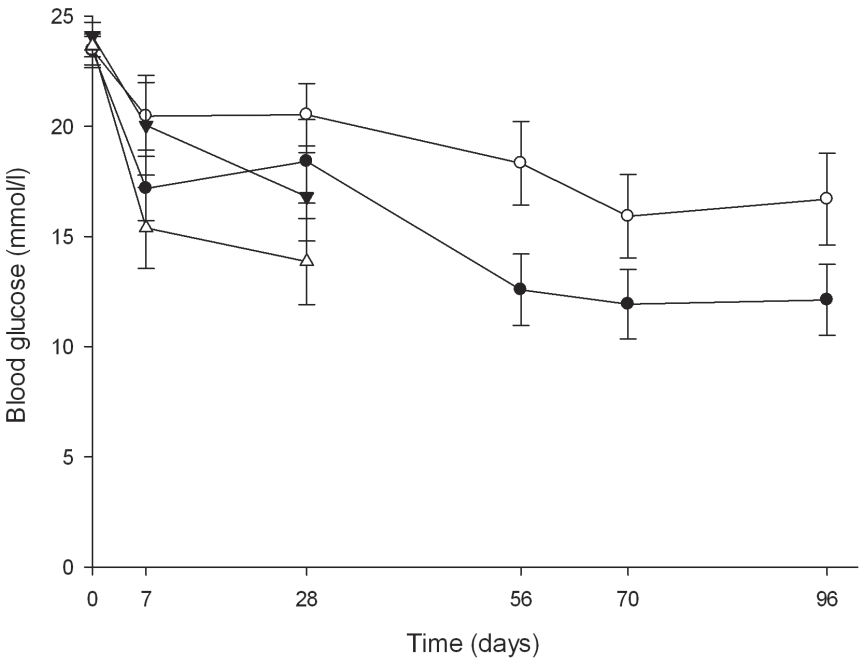

Figure 5. Blood glucose levels in STZ diabetic male C57BL/6AF1 mice transplanted with 150 fresh islets with administration of salicylate in the water (closed triangles) or regular water (open triangles). 2 way RM ANOVA with the factors being treatment and time; $p = 0.153$ for the effect of salicylate treatment on blood glucose, $p < 0.001$ for the effect of time on blood glucose, n = 11–14. Alternatively, STZ diabetic male C57BL/6AF1 mice were transplanted with 150 islets, which had been cultured for 72 h with 2 mmol/l salicylate (closed circles) or without salicylate (open circles). 2 way RM ANOVA; $p = 0.072$ for the effect of salicylate treatment on blood glucose, $p = 0.401$ for the effect of time on blood glucose, n = 12–14.
doi:10.1371/journal.pone.0077452.g005

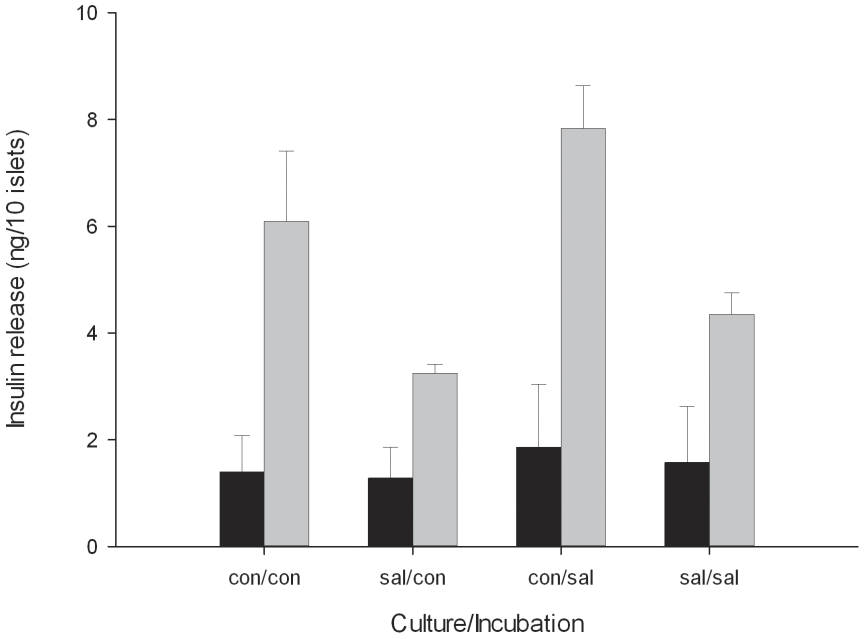

Figure 6. Insulin secretion in islets cultured with 2 mmol/l salicylate for 72 h and/or exposed to 2 mmol/l salicylate during the insulin release experiment (1 h at 1.7 mmol/l (black) and 1 h at 16.7 mmol/l glucose (grey)). p = 0.035, RM ANOVA with Student-Newman-Keuls (SNK) post hoc test, n = 5 separate experiments with triplicates.
doi:10.1371/journal.pone.0077452.g006

After culturing under control conditions (RPMI 1640 with 10% FCS) for 48 h, wild-type islets contained 20.5±7.2 ng insulin/islet and BIKK islets contained 14.3±3.1 ng insulin/islet (p = 0.4, t-test, n = 5–7). After culture in 33 mmol/l glucose, there was a tendency for the βIKK islets to contain less insulin (9.1±1.9 ng/ wild type islet versus 5.2±1.1 ng/βIKK islet, p = 0.09, n = 5–7). After exposure to 2.5 U/ml IL-1β, insulin content of wild-type islets was 8.1±1.6 ng/islet and in βIKK islets 10.5±2.5 ng/islet (p = 0.5, n = 5–7).

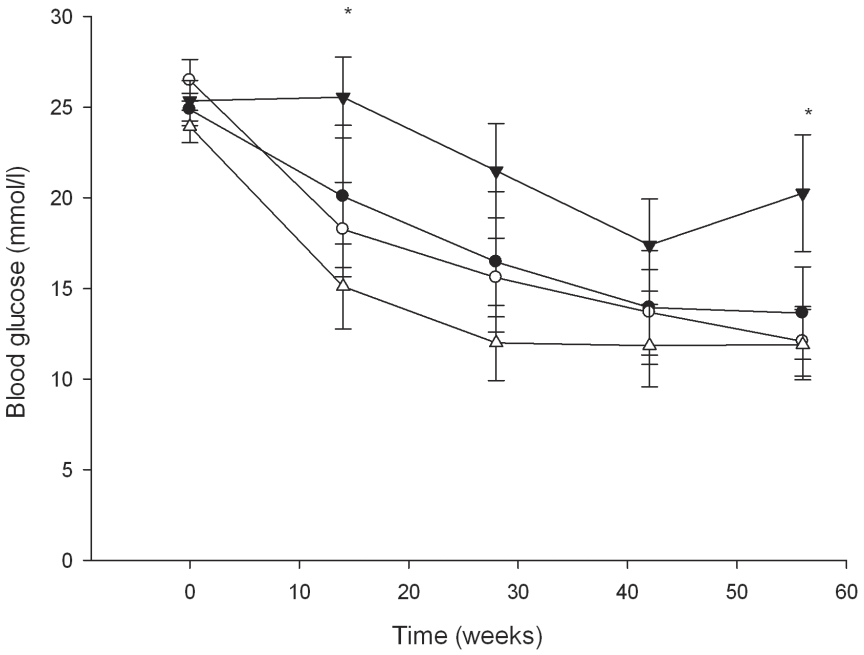

Figure 7. Blood glucose levels after transplantation of 150 islets from βISR mice (closed circles) or βIKK mice (closed triangles) and their respective wild-type littermates (open symbols) into STZ diabetic male C57BL/6AF1 recipients. n = 8–13. * = p<0.05, t-test vs wild-type littermates of βIKK mice, n = 12–13.
doi:10.1371/journal.pone.0077452.g007

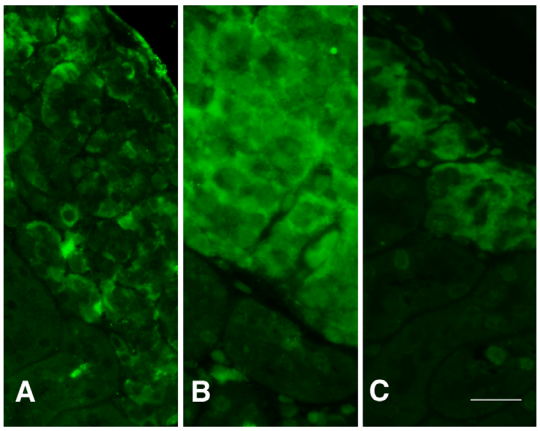

Figure 8. NF-κB localization in βIKK and wild-type islets 98 days after transplantation. In wild-type islets (panel A and C), the NF-κB localization is cytoplasmic whereas in βIKK islets (panel B) the localization is nuclear. Scale bar is 20 μm.
doi:10.1371/journal.pone.0077452.g008

Discussion

The surprising finding of this study is that neither chronic inhibition (βISR) nor activation (βIKK) of NF-κB in β-cells of transgenic mice led to abnormal metabolic phenotypes, indicating that in vivo β-cell function was either normal or at least compensated. This was supported by studies of islet function *in vitro* with islets from βIKK mice, in which dysfunction might have been expected. Indeed, results of measurements of GSIS, insulin content, insulin mRNA, and glucose oxidation did not differ from results in control wild-type mice. In addition, when cultured islets were exposed to IL-1β or to high glucose levels, no significant differences in insulin content between βIKK and control islets emerged.

Transplantation experiments were used to further challenge the βIKK islets. In this situation, the βIKK islets did a little less well, but it is impressive that a minimal number of 150 islets with activated NF-κB were able to cure 36% of the mice. We can conclude that chronic activation of NF-κB does not have a very damaging effect on transplanted islets. It may be that chronic activation of NF-κB in islets is different than its acute activation, or that NF-κB activities in β cells are less important than previously suggested.

Roles of NF-κB in islet transplantation appear to be complex. There is even disagreement about whether NF-κB is activated by the trauma of the isolation process; some find activation [21,39] while others do not [40]. Given that NF-κB activation does occur, there are questions about how damaging it is because both proapoptotic and antiapoptotic factors can be generated [13,16]. However, it has been suggested that inhibiting NF-κB prior to and immediately after islet isolation does improve islet transplantation outcome [17,21]. Nonetheless, there are a variety of death pathways that could be independent of NF-κB such as c-jun NH2-terminal kinases (JNKs) [40] and poly(ADP-ribose) polymerase [39]. In addition, there must be adaptive changes that occur over the time period NF-κB is activated. The acute changes seen after isolation may also be different than those produced through activation by an inducible or constitutive transgene. In addition to changes induced by the isolation process, more serious trauma is inflicted during the peritransplant period such as anoxic cell death [41]. Indeed it has been recently suggested that hypoxic conditions can determine whether NF-κB is pro- or anti- apoptotic [14].

A question addressed by this study is whether inhibition of NF-κB either by genetic or by pharmacological means might protect transplanted islets. In spite of the complexities outlined above, there was reason to think that NF-κB inhibition might protect islets after isolation and/or during the peritransplant period. The βISR mice were created to provide constitutive inhibition of NF-κB. Unexpectedly, transplantation of a marginal number of βISR islets did no better than control wild type mouse islets in a syngeneic model. It is entirely possible that an acute intervention might have provided protection not seen with our chronic model, as was reported recently by Rink et al [17]. The current study does however indicate that whatever cell death occurred in this transplant situation was independent of NF-κB. However it should be noted that the situation may be different in the case of allogeneic rejection, where inhibition of NF-κB has been shown to prolong graft survival [18,19]. Salicylate treatment provided an opportunity to test the effects of pharmacologically interfering with NF-κB. Salicylate is known to inhibit NF-κB and was previously shown to have antiapoptotic effects in human islets [11]. Treatment of recipient mice by addition of salicylate to drinking water provided no benefit, but culture of islets with salicylate prior to transplantation provided outcomes that came close to being significantly better ($p = 0.072$). Insight into the issue of acute versus chronic activation may be provided by a paper in which NF-κB was conditionally blocked in a transgenic model by treatment with doxycycline for three days [16]. This was followed by impressive protection of islets from cytokine-induced apoptosis and treatment with multiple low-dose streptozotocin. In a transplantation setting, it has been recently suggested that

Table 1. Insulin secretion from wild type (WT) and βIKK islets after culture for 48 h in 2.5 U/ml IL-1β, 33 mmol/l glucose or in control media.

Culture conditions (48 h)	**Control**		**2.5 U/ml IL-1β**		**33 mmol/l glucose**	
Glucose concentration during incubation (mmol/l)	**1.7**	**16.7**	**1.7**	**16.7**	**1.7**	**16.7**
WT islet insulin release (ng/10 islets/h)	2.4±0.6	16.5±5.8	5.4±2.2	9.2±2.1	12.7±1.0	28.0±5.7
βIKK islet insulin release (ng/10 islets/h) *(p value: t-test vs WT islets)*	3.2±1.1 (p = 0.57)	13.0±5.8 (p = 0.69)	7.3±2.5 (p = 0.75)	13.7±2.7 (p = 0.24)	11.6±3.3 (p = 0.51)	22.2±4.8 (p = 0.46)
Stimulation index WT islets	7.7±2.7		2.8±1.3		2.3±0.7	
Stimulation index βIKK islets (p *value: t-test vs WT islets)*	4.6±1.4 (p = 0.31)		3.9±1.8 (p = 0.69)		3.1±1.4 (p = 0.66)	

n = 5–7 separate experiments with triplicates.
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conditional knock-out of NF-κB in islets prior to isolation and culture can improve islet transplantation outcome in intraportally implanted islets [17]. This indicates that the viability of the islets prior to transplantation is particularly important. It is interesting to note that in our study, wild-type mice showed little NF-κB staining 8 wk after implantation, which may explain why chronic inhibition of NF-κB did not seem to have any beneficial effects.

In our model in which islets were pre-cultured with salicylate, there were trends towards improved transplantation outcomes. This indicates that inhibiting NF-κB for a short period prior to islet transplantation may be beneficial. Interestingly, islets that had been cultured with salicylate showed decreased GSIS but it is likely that this effect was reversible as after implantation, 64% of the animals with salicylate-treated islets cured. A salicylate-induced reduction in insulin secretion could also be related to the effects of salicylate on AMP protein kinase [42].

Beneficial effects were seen when an NF-κB inhibitor was administered immediately prior to the intraportal administration of islets [14], suggesting that acute inhibition has benefits. These studies indicate that while an acute inhibition may be beneficial in the few hours after implantation, a systemic chronic inhibition of NF-κB may be detrimental. In agreement with our study, McCall et al also showed that systemic administration of an NF-κB inhibitor over a period of weeks had no beneficial effects on islet transplantation outcome [43]. Inhibiting NF-κB systemically has been suggested to impair angiogenesis [44] and thus revascularization of the implanted islets may also be affected.

There is growing interest in the influence of NF-κB on β-cell function. Our in vitro studies on islets with activated NF-κB (βIKK) indicate that when evaluated for GSIS and various other parameters, they cannot be distinguished from normal islets. Unfortunately, because of breeding problems we were not able to study isolated islets from mice with inhibited NF-κB (βISR), but they had perfectly normal glucose tolerance. These results differ from those of Norlin et al, but their model was very different in that NF-κB activity was reduced by expression of a dominant active mutant IκBα under the Pdx1 promoter [45], which is turned on much earlier during β cell development. Hyperglycaemia seen in these mice may therefore reflect early embryonic effects of sustained NF-κB activity on islet development. Indeed, there was a 25% reduction in endocrine cell volume. The changes in islet function may have nothing to do with NF-κB inhibition because chronic hyperglycemia, even when very mild, is known to cause the type of β-cell dysfunction found in that study [46,47]. Inhibition of insulin secretion was found with a very different approach of acute inhibition with an inhibitor of IκBα phosphorylation (Bay 11-7082) [48]. This again highlights the likely difference between acute and chronic inhibition of NF-κB as a potential explanation for these divergent results.

In summary, the current study draws attention to the complexities of regarding the activation state of NF-κB and how this activation state regulates the physiological function of β-cells. Our findings suggest that pancreatic β-cells can adapt to both chronic activation and inhibition of this important transcription factor with normal or near normal β-cell function.

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Author Contributions

Conceived and designed the experiments: AK JC SBW SS GW. Performed the experiments: AK YG DC JHL BM AS. Analyzed the data: AK YG SBW GW. Wrote the paper: AK GW.

References

1. Padgett LE, Broniowska KA, Hansen PA, Corbett JA, Tse HM (2013) The role of reactive oxygen species and proinflammmmatory cytokines in type 1 diabetes pathogenesis. Ann N Y Acad Sci 1281: 16–35.
2. Rabinovitch A, Suarez-Pinzon WL (1998) Cytokines and their roles in pancreatic islet beta-cell destruction and insulin-dependent diabetes mellitus. Biochem Pharmacol 55: 1139–1149.
3. Mandrup-Poulsen T (1996) The role of interleukin-1 in the pathogenesis of IDDM. Diabetologia 39: 1005–1029.
4. Steer SA, Scarim AL, Chambers KT, Corbett JA (2006) Interleukin-1 stimulates beta-cell necrosis and release of the immmmunological adjuvant HMGB1. PLoS Med 3: e17.
5. Collier JJ, Fueger PT, Hohmeier HE, Newgard CB (2006) Pro- and antiapoptotic proteins regulate apoptosis but do not protect against cytokine-mediated cytotoxicity in rat islets and beta-cell lines. Diabetes 55: 1398–1406.
6. Giannoukakis N, Rudert WA, Trucco M, Robbins PD (2000) Protection of human islets from the effects of interleukin-1beta by adenoviral gene transfer of an Ikappa B repressor. J Biol Chem 275: 36509–36513.
7. Cardozo AK, Heimberg H, Heremans Y, Leeman R, Kutlu B, et al (2001) A comprehensive analysis of cytokine-induced and nuclear factor-kappa B-dependent genes in primary rat pancreatic beta-cells. J Biol Chem 276: 48879–48886.
8. Laybutt DR, Kaneto H, Hasenkamp W, Grey S, Jonas JC, et al (2002) Increased expression of antioxidant and antiapoptotic genes in islets that may contribute to beta-cell survival during chronic hyperglycemia. Diabetes 51: 413–423.
9. Kwon G, Corbett JA, Rodi CP, Sullivan P, McDaniel ML (1995) Interleukin-1 beta-induced nitric oxide synthase expression by rat pancreatic beta-cells: evidence for the involvement of nuclear factor kappa B in the signaling mechanism. Endocrinology 136: 4790–4795.
10. Heimberg H, Heremans Y, Jobin C, Leemans R, Cardozo AK, et al (2001) Inhibition of cytokine-induced NF-kappaB activation by adenovirus-mediated expression of a NF-kappaB super-repressor prevents beta-cell apoptosis. Diabetes 50: 2219–2224.
11. Zeender E, Maedler K, Bosco D, Berney T, Donath MY, et al (2004) Pioglitazone and sodium salicylate protect human beta-cells against apoptosis and impaired function induced by glucose and interleukin-1beta. J Clin Endocrinol Metab 89: 5059–5066.
12. Chang I, Kim S, Kim JY, Cho N, Kim YH, et al (2003) Nuclear factor kappaB protects pancreatic beta-cells from tumor necrosis factor-alpha-mediated apoptosis. Diabetes 52: 1169–1175.
13. Papaccio G, Graziano A, d'Aquino R, Valiante S, Naro F (2005) A biphasic role of nuclear transcription factor (NF)-kappaB in the islet beta-cell apoptosis induced by interleukin (IL)-1beta. J Cell Physiol 204: 124–130.
14. Chen C, Moreno R, Samikannu B, Bretzel RG, Schmitz ML, et al (2011) Improved intraportal islet transplantation outcome by systemic IKK-beta inhibition: NF-kappaB activity in pancreatic islets depends on oxygen availability. Am J Transplant 11: 215–224.
15. Mabley JG, Hasko G, Liaudet L, Soriano FG, Southan GJ, et al (2002) NFkappaB1 (p50)-deficient mice are not susceptible to multiple low-dose streptozotocin-induced diabetes. J Endocrinol 173: 457–464.
16. Eldor R, Yeffet A, Baum K, Doviner V, Amar D, et al (2006) Conditional and specific NF-kappaB blockade protects pancreatic beta cells from diabetogenic agents. Proc Natl Acad Sci U S A 103: 5072–5077.
17. Rink JS, Chen X, Zhang X, Kaufman DB (2012) Conditional and specific inhibition of NF-kappaB in mouse pancreatic beta cells prevents cytokine-induced deleterious effects and improves islet survival posttransplant. Surgery 151: 330–339.
18. Ding X, Wang X, Xue W, Tian X, Li Y, et al (2012) Blockade of the nuclear factor kappa B pathway prolonged islet allograft survival. Artif Organs 36: E21–E27.
19. Eldor R, Abel R, Sever D, Sadoun G, Peled A, et al (2013) Inhibition of Nuclear Factor-kappaB Activation in Pancreatic beta-Cells Has a Protective Effect on Allogeneic Pancreatic Islet Graft Survival. PLoS One 8: e56924.
20. Sorelle JA, Itoh T, Peng H, Kanak MA, Sugimoto K, et al (2013) Withaferin A inhibits pro-inflammatory cytokine-induced damage to islets in culture and following transplantation. Diabetologia 56: 814–824.
21. Takahashi T, Matsumoto S, Matsushita M, Kamachi H, Tsuruga Y, et al (2010) Donor pretreatment with DHMEQ improves islet transplantation. J Surg Res 163: e23–e34.
22. Fiorina P, Shapiro AM, Ricordi C, Secchi A (2008) The clinical impact of islet transplantation. Am J Transplant 8: 1990–1997.
23. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, et al (2000) Islet transplantation in seven patients with type 1 diabetes mellitus using a

glucocorticoid-free immmmunosuppressive regimen. N Engl J Med 343: 230–238.
24. Ryan EA, Paty BW, Senior PA, Bigam D, Alfadhli E, et al (2005) Five-year follow-up after clinical islet transplantation. Diabetes 54: 2060–2069.
25. Baeuerle PA, Baltimore D (1988) I kappa B: a specific inhibitor of the NF-kappa B transcription factor. Science 242: 540–546.
26. DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M (1997) A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature 388: 548–554.
27. Kopp E, Ghosh S (1994) Inhibition of NF-kappa B by sodium salicylate and aspirin. Science 265: 956–959.
28. Yuan M, Konstantopoulos N, Lee J, Hansen L, Li ZW, et al (2001) Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. Science 293: 1673–1677.
29. Goldfine AB, Fonseca V, Jablonski KA, Pyle L, Staten MA, et al (2010) The effects of salsalate on glycemic control in patients with type 2 diabetes: a randomized trial. Ann Intern Med 152: 346–357.
30. Goldfine AB, Fonseca V, Jablonski KA, Chen YD, Tipton L, et al (2013) Salicylate (salsalate) in patients with type 2 diabetes: a randomized trial. Ann Intern Med 159: 1–12.
31. Cai D, Frantz JD, Tawa NE, Jr., Melendez PA, Oh BC, et al (2004) IKKbeta/NF-kappaB activation causes severe muscle wasting in mice. Cell 119: 285–298.
32. Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, et al (2005) Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. Nat Med 11: 183–190.
33. Heitmeier MR, Scarim AL, Corbett JA (1997) Interferon-gamma increases the sensitivity of islets of Langerhans for inducible nitric-oxide synthase expression induced by interleukin 1. J Biol Chem 272: 13697–13704.
34. Heitmeier MR, Scarim AL, Corbett JA (1999) Prolonged STAT1 activation is associated with interferon-gamma priming for interleukin-1-induced inducible nitric-oxide synthase expression by islets of Langerhans. J Biol Chem 274: 29266–29273.
35. Gotoh M, Maki T, Kiyoizumi T, Satomi S, Monaco AP (1985) An improved method for isolation of mouse pancreatic islets. Transplantation 40: 437–438.
36. Montana E, Bonner-Weir S, Weir GC (1993) Beta cell mass and growth after syngeneic islet cell transplantation in normal and streptozocin diabetic C57BL/6 mice. J Clin Invest 91: 780–787.
37. Andersson A, Sandler S (1983) Viability tests of cryopreserved endocrine pancreatic cells. Cryobiology 20: 161–168.
38. Tillmar L, Carlsson C, Welsh N (2002) Control of insulin mRNA stability in rat pancreatic islets. Regulatory role of a 3′-untranslated region pyrimidine-rich sequence. J Biol Chem 277: 1099–1106.
39. Bottino R, Balamurugan AN, Tse H, Thirunavukkarasu C, Ge X, et al (2004) Response of human islets to isolation stress and the effect of antioxidant treatment. Diabetes 53: 2559–2568.
40. Abdelli S, Ansite J, Roduit R, Borsello T, Matsumoto I, et al (2004) Intracellular stress signaling pathways activated during human islet preparation and following acute cytokine exposure. Diabetes 53: 2815–2823.
41. Davalli AM, Scaglia L, Zangen DH, Hollister J, Bonner-Weir S, et al (1996) Vulnerability of islets in the immediate posttransplantation period. Dynamic changes in structure and function. Diabetes 45: 1161–1167.
42. Hawley SA, Fullerton MD, Ross FA, Schertzer JD, Chevtzoff C, et al (2012) The Ancient Drug Salicylate Directly Activates AMP-Activated Protein Kinase. Science.
43. McCall MD, Pawlick R, Shapiro AM (2011) Resveratrol fails to improve marginal mass engraftment of transplanted islets of Langerhans in mice. Islets 3.
44. Borthwick GM, Johnson AS, Partington M, Burn J, Wilson R, et al (2006) Therapeutic levels of aspirin and salicylate directly inhibit a model of angiogenesis through a Cox-independent mechanism. FASEB J 20: 2009–2016.
45. Norlin S, Ahlgren U, Edlund H (2005) Nuclear factor-{kappa}B activity in {beta}-cells is required for glucose-stimulated insulin secretion. Diabetes 54: 125–132.
46. Jonas JC, Sharma A, Hasenkamp W, Ilkova H, Patane G, et al (1999) Chronic hyperglycemia triggers loss of pancreatic beta cell differentiation in an animal model of diabetes. J Biol Chem 274: 14112–14121.
47. Laybutt DR, Glandt M, Xu G, Ahn YB, Trivedi N, et al (2003) Critical reduction in beta-cell mass results in two distinct outcomes over time. Adaptation with impaired glucose tolerance or decompensated diabetes. J Biol Chem 278: 2997–3005.
48. Hammar EB, Irminger JC, Rickenbach K, Parnaud G, Ribaux P, et al (2005) Activation of NF-kappaB by extracellular matrix is involved in spreading and glucose-stimulated insulin secretion of pancreatic beta cells. J Biol Chem 280: 30630–30637.