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ORIGINAL PAPER

Streptozotocin-resistant BRIN-BD11 cells possess wide spectrum of toxin tolerance and enhanced insulin-secretory capacity

Hui-Kang Liu · Jane T. McCluskey · Neville H. McClenghan · Peter R. Flatt

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Abstract Since streptozotocin (STZ) exhibits beta-cell toxicity, mediated through diverse mechanisms, multiple toxin resistance can be expected in insulin-secretory cells rendered STZ-resistant. RINm5F, but not all cell lines surviving STZ treatment, possess higher insulin content than native parental cells and additional tolerance against alloxan. To understand the impact of STZ tolerant cell selection on toxin resistance and insulin-secretory function, STZ-resistant BRIN-BD11 cells were generated by iterative acute exposure to 20 mM STZ. These cells, denoted BRINst cells, exhibited resistance to toxic challenges from STZ, H_2O_2, and ninhydrin. Insulin content and both glucose and arginine-stimulated insulin secretion were significantly enhanced in BRINst cells. The toxin-resistance of BRINst cells was gradually lost during continuous cultivation without STZ challenge. However, enhanced insulin secretory capacity at high passage in BRINst cells persisted. Although total SOD activity was decreased, catalase activity was increased and appeared to be important for the ninhydrin and STZ resistance of BRINst cells. This was associated with reductions of both STZ- and ninhydrin-induced DNA damage, although DNA repair was abolished. Further characterization of cells exhibiting multiple toxin tolerance and an enhanced insulin secretory function could provide useful lessons for understanding of beta-cell survival.

H.-K. Liu · J. T. McCluskey · N. H. McClenghan · P. R. Flatt
School of Biomedical Sciences, University of Ulster, Coleraine BT52 1SA, Northern Ireland, UK

H.-K. Liu (✉)
Division of Herbal Drugs and Natural Products, National Research Institute of Chinese Medicine, Taipei 112, Taiwan
e-mail: hk.liu@nricm.edu.tw

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Introduction

Streptozotocin (STZ, 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) is a compound comprising 1-methyl-1-nitrosourea with a D-glucose moiety at position C2 that specifically destroys pancreatic beta-cells when administered to experimental animals [1–3]. Studies of the diabetogenic actions of STZ have contributed significantly to understanding of the mechanisms of beta-cell destruction and appreciation of chemically induced type 1 diabetes in man induced by vacor, pentamidine, and possibly consumption of foods rich in nitroso-compounds [4–6]. Moreover, STZ has been used extensively to generate animal models of reduced beta-cell mass resembling both type 1 and 2 diabetes [7].

In contrast to the other commonly used chemical diabetogen, alloxan, the cytotoxic action of STZ appears to be more complicated involving multiple toxic pathways [8]. STZ uptake by the beta-cell glucose transporter 2 (GLUT2) is important. Thus, over-expression of GLUT2 in insulinoma-derived RIN cells significantly enhanced sensitivity of transfected cells toward STZ compared with non-transfected cells [9, 10]. Also, compared with other alkylating agents that produce similar DNA damage, GLUT2 transfected cells showed an increased sensitivity toward STZ [10]. However, results from other studies using GLUT2 transfected genetically engineered non-beta-cells that produce insulin, suggested that other factors might be involved [11]. Another important action of STZ is to inhibit glucokinase activity and gene expression in beta-cells consistent

with observed impairment of glucose-induced insulin secretion after acute STZ exposure [12].

Although superoxide generation by STZ has not been proven [13], generation of hydrogen peroxide and nitric oxide from STZ has been measured and suggested to increase oxidative and nitrosative stress leading to DNA damage and apoptosis in pancreatic beta-cells [14–16]. The STZ also possesses alkylating ability and it is believed that this relates to its nitrosourea moiety, particularly guanine position O6, which may play an important role in STZ-induced beta-cell death [17, 18]. Based on the hypothesis of polyADP-ribosylation-induced programmed cell death, chemical insults on DNA can either directly or indirectly introduce DNA strand breaks via base-excision repair mechanisms [17, 18]. Both types of break need poly(ADP-ribose) polymerase to act as a nick protector to prevent unexpected homologous recombination via polyADP-ribosylation with NAD as a substrate. This has been shown to cause NAD depletion that ultimately results in cell death [19, 20].

In order to avoid NAD depletion and cell death, self-defense mechanisms that might help pancreatic beta-cells counter cytotoxic challenge have been proposed. A variety of reactive oxygen species (ROS) scavengers have been shown to reduce STZ-induced beta-cell cytotoxicity by inclusion in cell incubations or by gene overexpression [2, 21–23]. However, the question remains as to whether such defense mechanisms would be induced in insulin-secreting cells exposed to toxin challenge. One experimental approach concerns the production of STZ-resistant cells and comparison of their characteristics with native parental cells. This strategy was used to demonstrate that naturally induced cell defense was important for cell resistance [7, 24–28]. STZ-resistant RIN cells became insensitive to both STZ and alloxan and also demonstrated increased cellular insulin content and glucose-induced insulin release [26, 29]. In addition, GLUT2 expression was decreased suggesting a potential explanation for the STZ resistance in these resistant cells [29].

In contrast with other toxin-resistant cells, insulin secretory function was impaired in BRIN-BD11 cells rendered resistant to stable alloxan analogue, ninhydrin [30]. In contrast to RINm5F cells, clonal BRIN-BD11 cells are more glucose- and nutrient-responsive coupled with higher levels of GLUT2 and glucokinase expression [31, 32]. In the present study, STZ-resistant BRIN-BD11 cells were generated and evaluated for elements of beta-cell stimulus-secretion coupling pathways and insulin-secretory function. Moreover, the resistant cells were assessed to see if either antioxidant enzyme status or DNA repair effectiveness were improved. BRIN-BD11 cells are useful for such studies, as they represent a well characterized and robust glucose-responsive clonal cell line that responds well with a wide range of agents affecting pancreatic beta cell function [31].

Materials and methods

Chemicals

Antibiotics (100 U/ml penicillin and 0.1 g/l streptomycin), fetal bovine serum (FBS), Hanks' balanced saline solution (HBSS), RPMI-1640 tissue culture medium (supplemented with 0.3 g/l L-glutamine), trypsin/EDTA (10×; 5.0 g trypsin (1:250), and EDTA 2.0 g/l normal saline) were purchased from Gibco (Paisley, Strathclyde, UK). DNAse I, DNA ladder (100 bp), primers, superscript one step (SSOS) kit, TBE buffer (10× stock) were obtained from Invitrogen (Paisley, UK). RANSOD kit was from Randox Laboratories (Ardmore, Antrim, UK). All other reagents were purchased from Sigma Chemical Company Ltd. (Poole, Dorset, UK) or BDH Chemicals Ltd. (Poole, Dorset, UK).

Cultivation of BRIN-BD11 cells and selection for streptozotocin (STZ) resistance

BRIN-BD11 cells were generated in our laboratory by electrofusion of normal rat beta cell with immortal RINm5F cells. The origin and characteristics of these cells are detailed elsewhere [31, 32]. BRIN-BD11 cells were cultured routinely with RPMI-1640 tissue culture medium containing 11.1 mM glucose and supplemented with 10% (v/v), fetal bovine serum, and 1% (v/v) 100 U/ml penicillin and 0.1 mg/l streptomycin and maintained at 37°C in an atmosphere of 5% CO_2 and 95% air using a LEEC incubator (Laboratory Technical Engineering, Nottingham, England). The procedure for selection of STZ-resistant (BRINst) cells involved culture of surviving cells following repeated acute exposures to STZ. Briefly, BRIN-BD11 cells were seeded into 24-multiwell plates at a density of 1.0×10^5 cells/well. After overnight attachment, cells were exposed for 60 min to freshly prepared 20 mM STZ at 37°C in Krebs Ringer Bicarbonate (KRB) buffer consisting of 115 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.28 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM Hepes, and 8.4% (w/v) $NaHCO_3$ supplemented with 0.1%(w/v) BSA and 1.1 mM D-glucose (pH 7.4). After exposure, treated cells were washed once and test medium was replaced by fresh growth medium. Viability (percentage of control viable cell number) was determined at 24 and 48 h after STZ exposure. A small aliquot of trypsinized cell suspension was used to determine total cell number under a haemocytometer with the aid of trypan blue stain. Cells surviving were propagated in culture to produce sufficient

number of cells for the next exposure. Cells surviving after 10 successive exposures were named STZ-tolerant cells (denoted BRINst cells). Aliquots were cryopreserved in liquid nitrogen prior to characterization.

Determination of DNA strand breaks

Evaluation of nuclei DNA strand breaks from cells directly after various toxin exposure was made by single-cell gel electrophoresis assay [33]. Briefly, platforms consisting of 1% standard agarose gel were produced using Dakin fully frosted microscope slides (Curtin Matheson, Houston, USA). 1×10^3 to 5×10^5 cells were resuspended in 1% pre-warmed (37°C) low melting agarose gel solution in phosphate buffer and rapidly applied to the platform described above to generate the second layer. The slides were incubated at 4°C for at least 1 h with cold lysis buffer, containing 2.5 M NaCl, 100 mM EDTA, and 10 mM Tris; pH 10.0 with 1% Triton X-100. Slides were then placed into the gel box (Bio-Rad, Richmond, CA, USA) for 20 min with freshly prepared cold electrophoresis buffer, containing NaOH (300 mM) and EDTA (1 mM) prior to electrophoresis at 4°C for 20 min at 25 volts, 300 mA. Slides were gently washed three times with neutralizing buffer, containing Tris (0.4 M), pH 7.5 and stained with ethidium bromide solution (20 μg/ml in phosphate buffer). Measurement of DNA damage was performed using an epi-fluorescence microscope (Nikon, Kingston, Surrey, UK) and software, Komet version 4.0 (Kinetic imaging, Liverpool, UK). The percentage of comet tail was used as the indicator of DNA damage.

Determination of DNA repair efficiency

In order to further investigate DNA repair efficiency in both BRIN-BD11 cells and BRINst cells, STZ challenged cells were continuously cultured with normal growth medium in the absence of STZ. Cells cultivated at 0, 4, 8, 24 h post-treatment were taken for the measurement of DNA damage, as described above. DNA repair efficiency represents the percentage of reduction of initial DNA damage (i.e. DNA damage at 0 h post-treatment of STZ = 100%).

Measurement of total superoxide dismutase (SOD) activity

Cells (4×10^7) cells were disrupted by sonication and cytoplasmic protein fractions were collected by consecutive centrifugations comprising 1,000*g* (10 min), 10,000*g* (10 min), and 100,000*g* (1 h), respectively. Pellet from each set of centrifugation was discarded. The last supernatant containing cytoplasmic protein fractions was collected and protein content was measured by Bradford assay. Total SOD activity was determined using the RANSOD assay kit (Randox, Crumlin, UK) based on the protocol designed for COBAS FARA II (Roche, Rankin Biomedical Corp., USA).

Measurement of catalase (CAT) activity

Cells (7×10^7 to 8×10^7) were disrupted by three cycles of freezing and thawing. After centrifugation at 1,000*g* for 10 min at 4°C, the supernatant was used directly to measure protein by Bradford assay and catalase enzyme activity. The reaction was started by addition of H_2O_2 and absorbance was recorded by spectrophotometry at wavelength of 240 nm.

Insulin secretion and cellular insulin content

Cells were trypsinized and seeded at a density of 1.5×10^5 cells/well in 24-multiwell plates. Following an overnight attachment at 37°C, cells were pre-incubated in KRB containing 1.1 mM glucose for 40 min at 37°C. Test incubations were performed in the same type of buffer supplemented with glucose and other agents as indicated in the Figures. Cells extracted using 500 μl of acid ethanol (1.5% (v/v) HCl, 75% (v/v) ethanol, and 23.5% (v/v) H_2O) were used to measure cellular insulin content. Samples were stored at –20°C until determination of insulin by radioimmunoassay [34].

Measurement of gene expression

Total RNA was extracted using TRI-reagent according to the manufacturer's instructions. The pelleted RNA was dissolved in distilled water, quantified by spectrophotometry and stored at –70°C. Total RNA (100 ng) was used as template and the RT-PCR followed the protocol given in the Gibco one-step RT-PCR kit. Sequence of primers for amplification of specific genes are shown in Table 1. Once the reaction was completed, RT-PCR products were separated by 2% agarose gel, visualized under UV box (Bio-Rad, Richmond, CA, USA) and photographed using a digital camera controlled by the Grab-It program for PC.

Table 1 Sequences of primers for specific genes investigated

5′-GGCAGCTATGTGAGAGOC-reverse
5′-GTTCCGAGGCCGCCGCGCGT-forward
5′-GTCCCCATATTGATGGAC-reverse
5′-AAGGGAACTACATCGTAGGA-forward
5′-CATTGGCGGTCTTCATAGTA-reverse
5′-CATTGCTGGAAGAAGCGTATCAG-forward
5′-GAGACCTTCTGCTCAGTCGAC-reverse
5′-CATCAGGAGAATGGCAAGAA-forward
5′-CCGCAGGAAGGTAAAGAGC-reverse
5′-TGCCCAGGCTTTTGTCAAACAGCACCTT-forward
5′-CTCCAGTGCCAAGGTCTGAA-reverse
5′-CTGAGGAGAGCAGCGGTCGT-forward
5′-CTTGGCCAGCGCCTCGTGGT-reverse
5′-CTCGCTGGGAACGCTGGAACA-forward
5′-GCTTTGGTGGATTTCATCCACGG-reverse

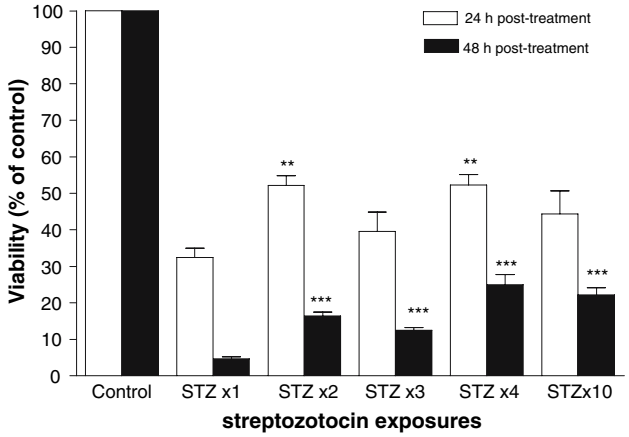


Fig. 1 Tolerance of BRIN-BD11 cells in response to STZ (20 mM) was improved after 10 consecutive exposures. Viability of STZ treated BRIN-BD11 cells at 24 and 48 h was measured. Values are percentage control expressed as mean ± SEM ($n = 4$). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the viability of cells after the first exposure under the same experimental conditions

Statistics

The significance of various treatments was determined by the unpaired Student's *t*-test. The results are expressed as mean ± S.E.M. Differences were considered significant if $P < 0.05$.

Results

Induction of resistance to streptozotocin with consecutive acute exposures

Preliminary experiments revealed that acute exposure of BRIN-BD11 cells to 20 mM STZ resulted in extensive cell death but survival of small population of cells. Accordingly this concentration was selected to generate streptozotocin-tolerant cells. The results of 10 consecutive exposures to STZ are depicted in Fig. 1. Significant enhancement of cell viability after 10 consecutive exposures (STZx10) was evident at 48 h ($P < 0.001$). The viability of STZx10 was increased 4.7-fold ($P < 0.001$) compared with the first exposure (STZx1) at 48 h incubation.

Multiple resistance to STZ, H_2O_2, or ninhydrin in streptozotocin-tolerant BRINst cells

The enhanced resistance in low passage BRINst cells toward acute exposure to streptozotocin, hydrogen peroxide or ninhydrin was demonstrated. As shown in Fig. 2, the viability of low passage BRINst cells at 24 h post-treatment of acute exposure to 10 and 20 mM streptozotocin increased 1.3-fold ($P < 0.05$) and 1.4-fold ($P < 0.05$), respectively, when compared with control BRIN-BD11 cells (Fig. 2A). Enhanced viability of low passage BRINst cells were also observed following exposure to hydrogen peroxide at concentrations of 0.1 mM (1.9-fold; $P < 0.001$) and 1 mM (3.8-fold; $P < 0.01$) (Fig. 2B). The viability of low passage BRINst cells was also significantly increased after exposure to ninhydrin at concentrations of 0.05 mM (2.4-fold; $P < 0.001$) and 0.1 mM (1.5-fold; $P < 0.05$) (Fig. 2C).

Enhancement of insulin secretory capacity in both low and high passage BRINst cells

Improved insulin secretory function was observed after continuous acute exposures to 20 mM STZ. As shown in Fig. 3A, insulin secretion in low passage BRINst cells was greater than native BRIN-BD11 cells following acute exposure to glucose at either 1.1 mM (2.9-fold; $P < 0.001$) or 16.7 mM (2.8-fold; $P < 0.001$). In addition, there was a 1.8-fold increase ($P < 0.01$) of insulin secreted after stimulation with arginine (20 mM) or extracelluar calcium (7.68 mM). Responses to KCl (30 mM) and alanine (20 mM) were unchanged or slightly depressed when considered in terms of cellular insulin content.

As shown in Fig. 3B, the enhanced of insulin secertory capacity of BRINst cells persisted over at least 10 passages of continuous culture in the absence of STZ. Glucose stimulated insulin secretion was increased 5.3-fold in high passage BRINst cells after exposure to either 1.1 or 16.7 mM glucose ($P < 0.001$). In addition, insulin secretion was enhanced in response to 20 mM alanine (2.6-fold;

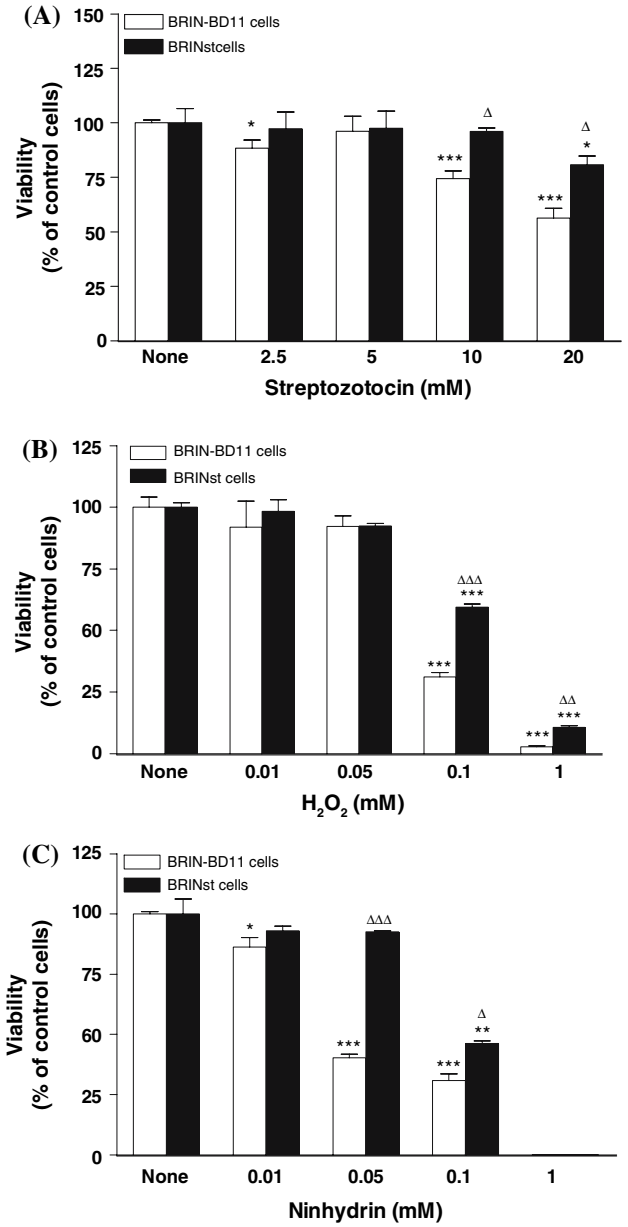


Fig. 2 BRINst cells exhibit multiple resistance to STZ, H_2O_2, or ninhydrin. Both BRIN-BD11 cells and BRINst cells were acutely exposed to (**A**) STZ, (**B**) H_2O_2, and (**C**) Ninhydrin at the concentrations indicated. Values are viability (percentage control) expressed as mean ± SEM ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control conditions. $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$, $^{\Delta\Delta\Delta}P < 0.001$ compared with BRIN-BD11 cells at the same toxin concentration. It should be noted that the viability of STZ treated cells were measured at 24 h post-treatment

$P < 0.001$), 20 mM arginine (3.6-fold; $P < 0.05$), 30 mM KCl (1.8-fold; $P < 0.001$), and 7.68 mM extracellular Ca^{2+} (2-fold; $P < 0.001$). Further analysis of glucose responsiveness in low and high passage BRINst cells suggested that low passage BRINst cells secreted significantly ($P < 0.001$) more insulin as a proportion of cellular insulin content (Fig. 3C).

Modulation of genes associated with glucose sensing and insulin production in BRINst cells

As shown in Fig. 4, comparison of low passage BRINst cells with control BRIN-BD11 cells suggested a significant up-regulation of PDX-1 and GLUT2 along with slight reduction of glucokinase and insulin mRNA level. On the other hand, in parallel with greater insulin content, an up-regulation of insulin and glucokinase was observed in high passage BRINst cells. This contrasted with an apparent down-regulation of GLUT2.

Antioxidant enzyme activities in BRINst cells

Since, antioxidant enzymes play important roles as free radical scavengers, their enzyme activity and the gene expression were compared between BRINst cells and native BRIN-BD11 cells. As shown in Fig. 5, there was a significant decrease in total superoxide dismutase activity (58%; $P < 0.001$) but a significant increase in the activity of catalase (1.3-fold; $P < 0.001$) in BRINst cells. Gene analysis indicated a modest up-regulation of CuZnSOD but no changes in other genes.

BRINst cells exhibit decreased STZ and ninhydrin-induced DNA damage but lack of DNA repair

Comparison of the sensitivity of the BRINst cells and native BRIN-BD11 cells toward streptozotocin, ninhydrin, and hydrogen peroxide-induced DNA damage was evaluated. After exposure to 20 mM streptozotocin, the majority of low passage BRINst cells and native BRIN-BD11 cells carried high levels of DNA damage (Fig. 6A). However, significantly lower percentage of DNA tail was found in the BRINst cell population in comparison with BRIN-BD11 cells ($P < 0.001$). In addition, a significant reduction ($P < 0.001$) of ninhydrin-induced DNA damage was observed in BRINst cells. DNA damage in BRINst cells and BRIN-BD11 cells was similar following acute exposure to H_2O_2 (0.05 mM). The reduction of DNA damage during prolonged culture after toxin-treatment might also reflect DNA repair. Therefore, DNA damage of both native BRIN-BD11 cells and low passage BRINst cells was measured at 0, 4, 8, and 24 h after acute exposure to STZ (20 mM). As shown in Fig. 6B, both cell types were unable

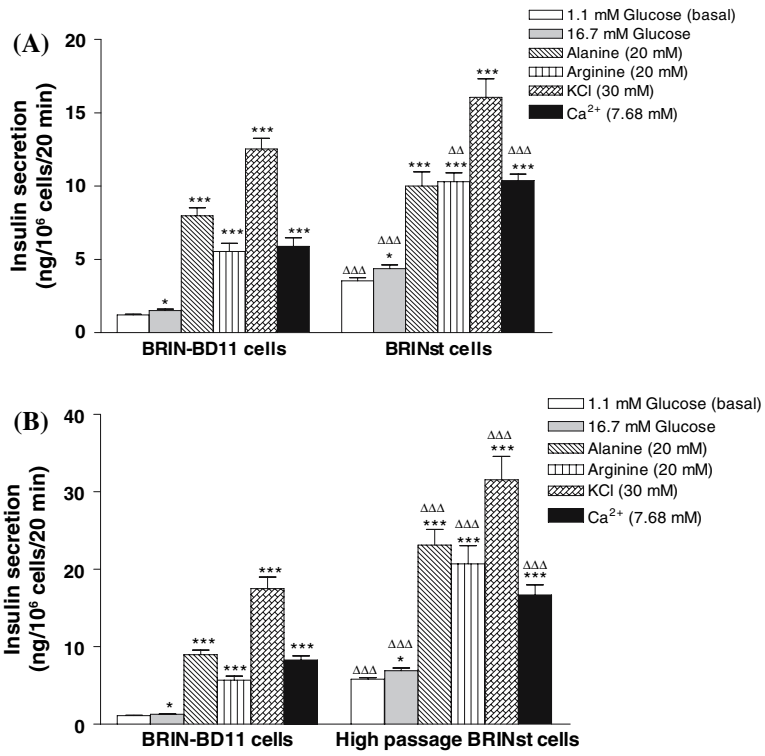


Fig. 3 Enhancement of insulin secretory capacity in both low and high passage BRINst cells. Insulin release from (**A**) Low and (**B**) High passage of BRINst cells in response to various secretagogues. (**A**) Values are mean ± SEM ($n = 4$). $^{*}P < 0.05$, $^{***}P < 0.001$ compared with respective effect at 1.1 mM glucose, respectively. $^{\Delta\Delta}P < 0.01$, $^{\Delta\Delta\Delta}P < 0.001$ compared with BRIN-BD11 cells, corresponsively. (**B**) Values are mean ± SEM ($n = 4$). $^{*}P < 0.05$, $^{***}P < 0.001$ compared with respective effect at 1.1 mM glucose, respectively. $^{\Delta\Delta\Delta}P < 0.001$ compared with BRIN-BD11 cells, corresponsively. (**C**) Basal insulin output and cellular insulin content were measured and data are expressed as ratio to insulin content to assist interpretation of insulin secretory responses. Values are mean ± SEM ($n = 8$). $^{*}P < 0.05$, $^{***}P < 0.001$ compared with BRIN-BD11 cells, corresponsively

(C)

		Basal Insulin secretion (ng/10^6 cells)	Cellular Insulin (ng/10^6 cells)	Insulin secretory response (% of insulin content)
Low Passage	**BRIN-BD11 cells**	1.2±0.1	67.8±4.0	1.8±0.1
	BRINst cells	3.5±0.2 ***	76.5±1.5 *	4.6±0.3 ***
High Passage	**BRIN-BD11 cells**	1.1±0.0	68.9±0.1	1.6±0.1
	BRINst cells	5.8±2.0 ***	318.6±3.2 ***	1.8±0.1

to reverse DNA damage caused by STZ treatment during 24 h of prolonged culture.

High passage BRINst cells lose toxin resistance

High passage BRINst cells were used to evaluate the stability of toxin resistance. As shown in Fig. 7A, high passage BRINst cells exhibited loss of tolerance to ninhydrin. In contrast, STZ resistance was reduced but remained superior to native BRIN-BD11 cells ($P < 0.01$). On the other hand, high passage BRINst cells exhibited better tolerance towards acute H_2O_2 exposure compared with native BRIN-BD11 cells ($P < 0.001$). Total SOD activity in high passage BRINst cells remained at a comparable level to native BRIN-BD11 cells (Fig. 7B). Furthermore, toxin induced DNA damage in high passage BRINst cells was not reduced compared with low passage BRINst cells (Fig. 7C). When assessing DNA repair efficiency by measuring the level DNA damage after acute STZ treatment, there was no sign of decreased DNA damage. Thus the abolishment of DNA repair appeared to remain in high passage BRINst cells (Fig. 7D).

Disscussion

Toxin-resistant insulin-secreting cells have been produced from INS cells and RIN cell lines, to generate cytokine-, alloxan-, or STZ-resistant daughter cells lines [26, 27]. In this study, glucose-responsive BRIN-BD11 cells were subjected to up to 10 iterative STZ exposures, rendering BRINst cells that were resistant to STZ as well as ninhydrin and hydrogen peroxide. Since ninhydrin is a generator

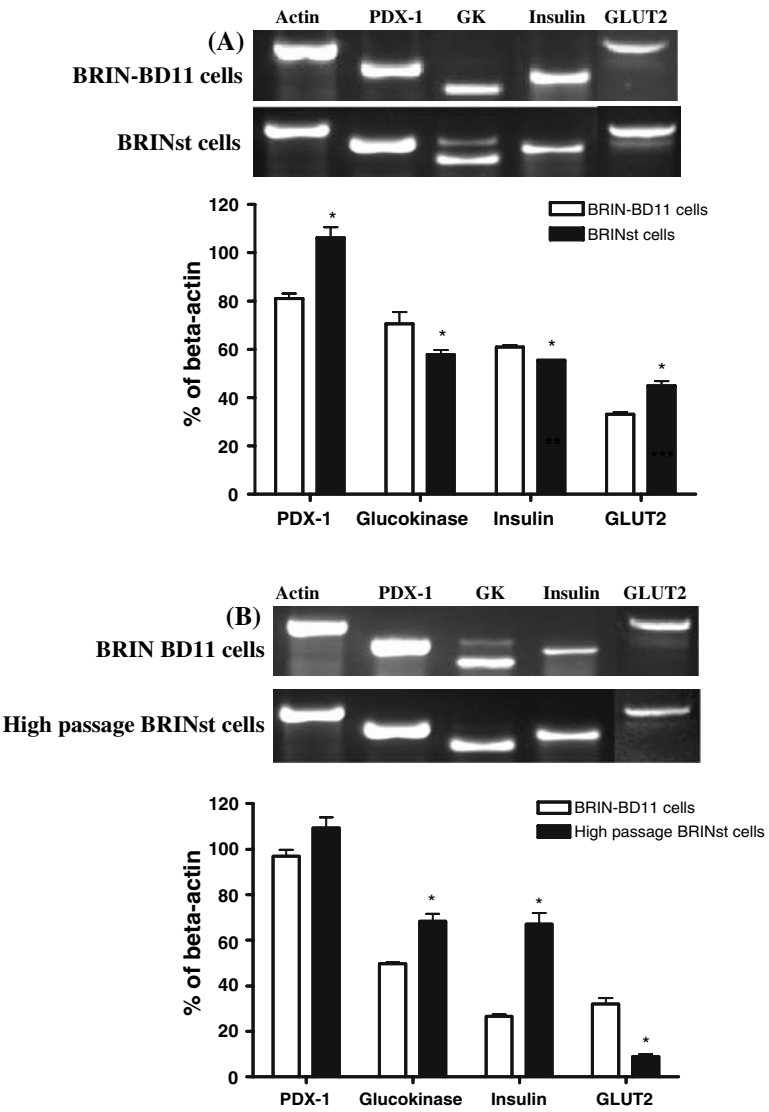


Fig. 4 Genes associated with glucose sensing and insulin production in BRINst cells. mRNA profile of beta-cell markers from low passage (**A**) and high passage (**B**) BRINst cells were evaluated by RT-PCR and compared with same passage of native BRIN-BD11 cells. Values are mean ± SEM ($n = 2$). $^{*}P < 0.05$ compared with the BRIN-BD11 cells, corresponsively

of hydroxyl radicals [35] whereas STZ exhibits multiple cytotoxic actions involving generation of ROS, lipid peroxidation, methylation of DNA, and induction of apoptosis [1, 3, 18, 36], BRINst cells may possess multiple defense mechanisms providing a wider spectrum of protection.

STZ-induced impairment of beta-cell function has been extensively reported in both in vivo and in vitro studies. Exposure to STZ, depending on dose, can lead to hyperglycemia and severe hypoinsulinemia [2] and in beta-cells to inhibition of glucokinase, hexokinase and aconitase activities, down-regulation of GLUT2 expression and defective insulin secretion [11, 12, 16, 37]. Since ninhydrin-tolerant BRIN-BD11 (BRINnt) cells exhibited decreased cellular insulin content and secretory function [30], the earlier report of improved insulin-secretory function of STZ-resistant RINm5F cells was surprising [24, 29]. However, STZ-tolerant BRIN-BD11 cells also exhibited a significant enhancement of secretagogue-induced insulin release. The mechanism underlying this effect is diverse and includes upregulation of genes for PDX-1, GLUT2, glucokinase, and insulin as well as enhancement of cellular insulin content.

The superior insulin-secretory characteristics of BRINst cells were retained following high passage in media without STZ. The suggestion of lower GLUT2 expression in these cells is intriguing. Several studies have demonstrated the importance of GLUT2 in mediating pancreatic beta-cell cytotoxicity of STZ [9]. Transportation of STZ via GLUT2 has been proposed to account for the greater diabetogenic action of STZ compared with other alkylating agents inducing similar DNA damage, such as *N*-methyl-*N*-nitrosourea (MNU) [38]. Enhanced STZ resistance was observed also in insulin-secreting RIN cells exhibiting down-regulation of GLUT2 which is

(A)

	Enzyme activities	
	SOD (U/mg protein)	Catalase (μU/mg protein)
BRIN-BD11 cells	33.5 ± 0.7	3.9 ± 0.2
BRINst cells	19.4 ± 1.2 ***	5.2 ± 0.1 ***

(B)

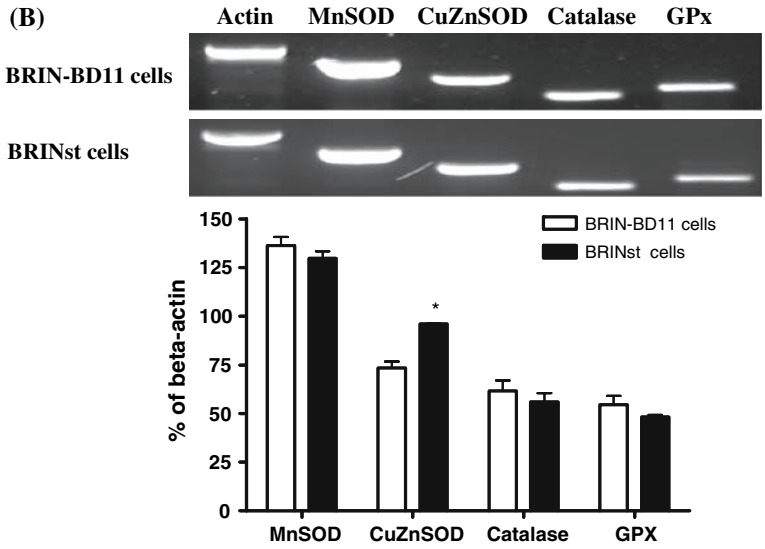


Fig. 5 Antioxidant enzyme activities in BRINst cells. (**A**) Total SOD and catalase enzyme activities of BRIN BD11 cells and BRINst cells. Values of enzyme activities are mean ± SEM (n = 8–10). ***$P < 0.001$ when compared with BRIN-BD11 cells. (**B**) mRNA levels of MnSOD, CuZnSOD , catalase, and glutathione peroxidase (GPx) were measured by RT-PCR and the arbitrary unit of the PCR product compared with expression of beta-actin. Values are presented as mean ± SEM (n = 2). *$P < 0.05$ when compared with BRIN-BD11 cells

required for effective STZ transport and toxicity in beta cells [10, 39]. Such observations fit with the present intact viability and functionality of high passage BRINst cells. On the other hand, low passage BRINst cells exhibited STZ resistance and normal glucose sensing with no signs of GLUT2 down-regulation. The results, therefore, suggest that other factors are also important for the diabetogenic actions of STZ.

Generation of ROS from STZ is controversial due to absence of STZ-induced chemiluminescence in pancreatic islets and lack of benefit of free radical scavengers in the development of STZ-induced diabetes [13, 40]. However, generation of hydrogen peroxide by STZ was detected in islets [15]. An increase of nitrate/nitrite content, an indication of nitric oxide generation, was also observed after STZ exposure due to spontaneous liberation [41] or metabolism following cellular STZ uptake [23]. Incubation with antioxidant enzymes, transfection of antioxidant enzyme genes and use of NO scavengers have been reported also to alleviate the cytotoxic effects of STZ and other ROS and NO donors, such as cytokines and nitrosourea compounds [23, 42–44]. Accordingly, elevated catalase activity in BRINst cells could partly reflect resistance to STZ, ninhydrin, and hydrogen peroxide. The importance of up-regulation of CuZnSOD in BRINst cells was unclear, but was similarly observed in ninhydrin-tolerant BRINnt cells [30]. Except for SOD and catalase, the thioredoxin family possesses both anti-apoptosis and antioxidant properties that can be induced in INS-1 and βTC6-F7 cells in response to cytokines, STZ, and alloxan [21]. The involvement of this family in BRINst cells remains to be elucidated.

A reduction of ninhydrin or STZ-induced DNA strand breaks was found in BRINst cells. Alloxan-mediated DNA strand breaks in rat islets has been associated with the generation of hydrogen peroxide [45]. Modification of pyrimidine-bases by alloxan has also been suggested and was reduced by catalase but not SOD [46, 47]. Decrease of ninhydrin-induced DNA damage could be the result of enhanced catalase activity in BRINst cells. On the other hand, STZ-induced DNA damage has been attributed to its properties as a hydrogen peroxide generator [10, 15], nitric oxide donor [48], and strong alkylating reagent which induces the methylation of DNA at guanines

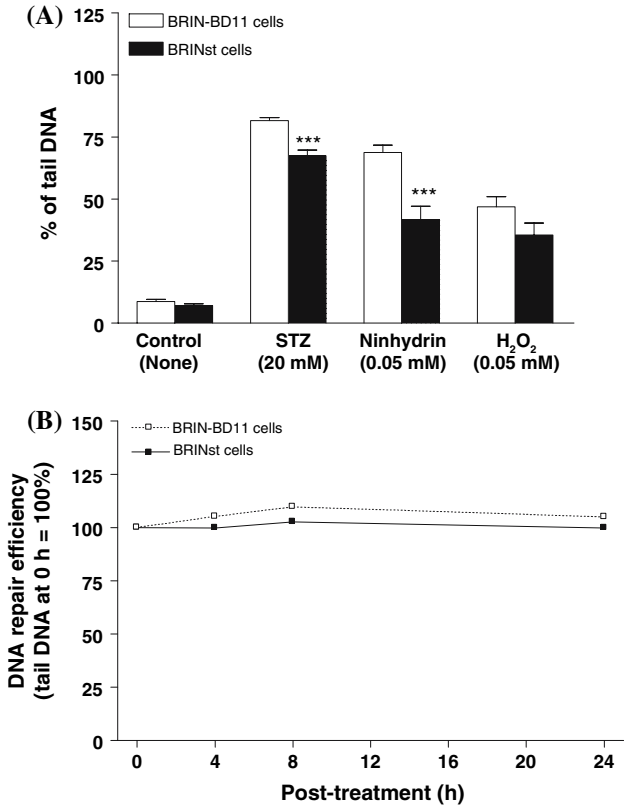
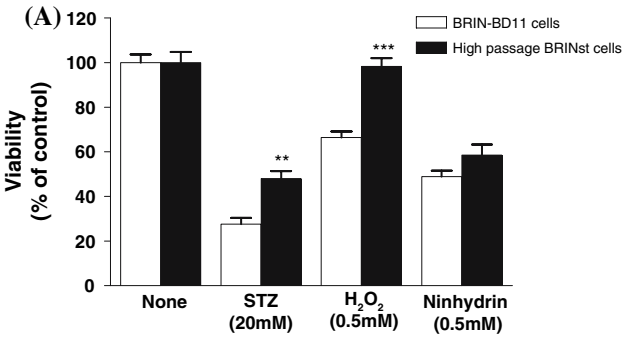


Fig. 6 BRINst cells ameliorated STZ and ninhydrin-induced DNA damages but abolished DNA repair after STZ treatment. (**A**) Both BRIN-BD11 cells and BRINst cells were acutely exposed to STZ, ninhydrin or hydrogen peroxide. Data are mean ± SEM (50 cells per condition over two experiments). ***$P < 0.001$ when compared with BRIN-BD11 cells under the same condition. (**B**) DNA repair at various time intervals after STZ exposure (time zero). Data are mean ± SEM (50 cells per condition over two experiments) and expressed as percentage average tail DNA at 0 h for each cell group

directly without the inhibition by ROS scavengers [18, 49]. Thus, it was not surprising that STZ-induced DNA damage was not totally restored in the present study. Although catalase activity was linked to reduction of DNA damage in BRINst cells, this was not the case after high passage in absence of STZ even though catalase activity was elevated. Other cell defense factors appear act in concert with catalase to provide a full protection in BRINst cells.

In terms of DNA repair, there were no signs that this occurred to any significant extent in STZ-treated BRIN-BD11 cells, regardless of toxin resistance. In conclusion, these data indicate that STZ-based selection of resistant beta-cell line can be used to generate a multiple toxin resistant cell lines exhibiting enhanced insulin secretory function. Further characterization of such cells could provide useful lessons for both understanding of beta-cell survival and future cell-based diabetes therapy.



(B)

	Enzyme activities	
	SOD (U/mg protein)	Catalase (U/mg protein)
BRIN-BD11 cells	27.2 ± 1.4	3.8 ± 0.4
High passage BRINst cells	27.1 ± 1.6	7.2 ± 0.4 *

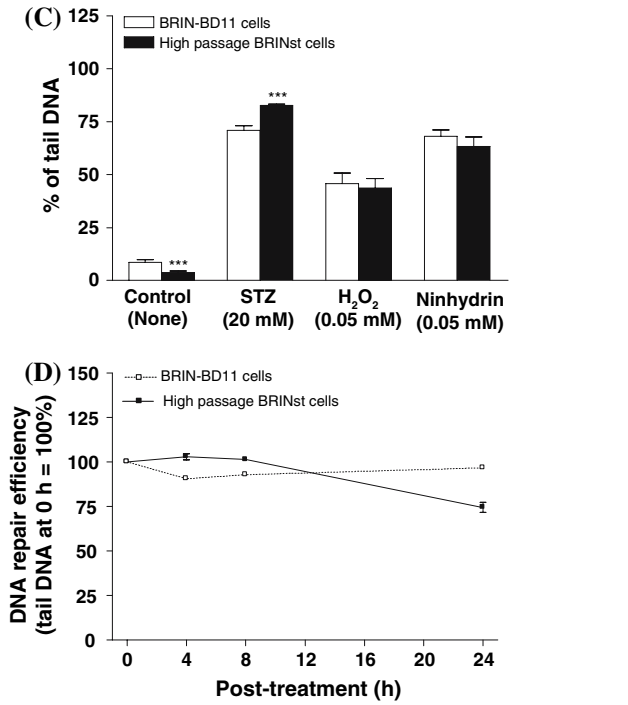


Fig. 7 Restoration of insulin secretory responsiveness in high passage BRINst cells plus accompanying loss of toxin resistance and cellular defense. (**A**) Toxin tolerance. Values are percentage control expressed as mean ± SEM ($n = 4$). (**B**) Antioxidant enzyme activity. Values of enzyme activities are mean ± SEM ($n = 8$–10). (**C**) DNA damage. Data are mean ± SEM (50 cells per condition over two experiments). (**D**) DNA repair. Data are mean ± SEM (50 cells per condition over two experiments). BRINst cells were continuously cultured for 10 passages without STZ challenge. Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared with parental BRIN-BD11 cells

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References

1. J.W. Baynes, in: *Drugs, Diet, and Disease*, ed by P.R. Flatt, C. Ioannides, (Ellis Horwood Limited, 1995)
2. P.R. Flatt, O. Shibier, C. Ioannides, S.K. Swanston Flatt, Exp. Clin. Endocrinol. **93**, 193–202 (1989)
3. C.C. Rerup, Pharmacol. Rev. **22**, 485–518 (1970)
4. P. Bouchard, P. Sai, G. Reach, I. Caubarrere, D. Ganeval, R. Assan, Diabetes **31**, 40–45 (1982)
5. T. Helgason, M.R. Jonasson, Lancet **2**, 716–720 (1981)
6. J.H. Karam, P.A. Lewitt, C.W. Young, R.E. Nowlain, B.J. Frankel, H. Fujiya, Z.R. Freedman, G.M. Grodsky, Diabetes **29**, 971–978 (1980)
7. C.J. Bailey, P.R. Flatt, in: *Textbook of Diabetes*, ed. by J.C. Pickup, G. Williams (Blackwell Science, Osney Mead, 2003)
8. F. Lally, A.J. Bone, in *Textbook of Diabetes*, ed. by J.C. Pickup, G. Williams (Blackwell Science, Osney Mead, 2003)
9. M. Elsner, B. Guldbakke, M. Tiedge, R. Munday, S. Lenzen, Diabetologia **43**, 1528–1533 (2000)
10. W.J. Schnedl, S. Ferber, J.H. Johnson, C.B. Newgard, Diabetes **43**, 1326–1333 (1994)
11. B.E. Tuch, S. Beynon, M.T. Tabiin, R. Sassoon, R.J. Goodman, A.M. Simpson, J Autoimmun. **10**, 239–244 (1997)
12. C. Park, J.R. Kim, J.K. Shim, B.S. Kang, Y.G. Park, K.S. Nam, Y.C. Lee, C.H. Kim, Arch. Biochem. Biophys. **362**, 217–224 (1999)
13. K. Asayama, D. English, A.E. Slonim, I.M. Burr, Diabetes **33**, 160–163 (1984)
14. K.S. Saini, C. Thompson, C.M. Winterford, N.I. Walker, D.P. Cameron, Biochem. Mol. Biol. Int. **39**, 1229–1236 (1996)
15. N. Takasu, I. Komiya, T. Asawa, Y. Nagasawa, T. Yamada, Diabetes **40**, 1141–1145 (1991)
16. J. Turk, J.A. Corbett, S. Ramanadham, A. Bohrer, M.L. McDaniel, Biochem. Biophys. Res. Commun. **197**, 1458–1464 (1993)
17. D.M. Kokkinakis, M.A. von Wronski, T.H. Vuong, T.P. Brent, S.C. Schold Jr., Br. J. Cancer **75**, 779–788 (1997)
18. M. Murata, A. Takahashi, I. Saito, S. Kawanishi, Biochem. Pharmacol. **57**, 881–887 (1999)
19. N.A. Berger, Radiat. Res. **101**, 4–15 (1985)
20. H. Okamoto, Biomed. Biochim. Acta. **44**, 15–20 (1985)
21. A. Bast, G. Wolf, R. Oberbaumer, R. Walther, Diabetologia **45**, 867–876 (2002)
22. H. Chen, E.C. Carlson, L. Pellet, J.T. Moritz, P.N. Epstein, Diabetes **50**, 2040–2046 (2001)
23. K.D. Kroncke, K. Fehsel, A. Sommer, M.L. Rodriguez, V. Kolb-Bachofen, Biol. Chem. Hoppe. Seyler. **376**, 179–185 (1995)
24. K. Bloch, P. Vardi, Int. J. Exp. Diabetes Res. **1**, 233–237 (2001)
25. K.O. Bloch, M. Vorobeychik, K. Yavrians, P. Vardi, Biochem. Pharmacol. **65**, 1797–1805 (2003)
26. K.O. Bloch, R. Zemel, O.V. Bloch, H. Grief, P. Vardi, Int. J. Exp. Diabetes Res. **1**, 211–219 (2000)
27. G. Chen, H.E. Hohmeier, R. Gasa, V.V. Tran, C.B. Newgard, Diabetes **49**, 562–570 (2000)
28. G. Chen, H.E. Hohmeier, C.B. Newgard, J. Biol. Chem. **276**, 766–772 (2001)
29. K. Bloch, P. Vardi, Diabetes Metab. Res. Rev. **21**, 253–261 (2005)
30. Y. Abdel-Wahab, H.-K. Liu, N.H. McClenaghan, P.R. Flatt, Diabetic Med. **23**, 33–34 (2006)
31. N.H. McClenaghan, C.R. Barnett, E. Ah-Sing, Y.H. Abdel-Wahab, F.P. O'Harte, T.W. Yoon, S.K. Swanston-Flatt, P.R. Flatt, Diabetes **45**, 1132–1140 (1996)
32. N.H. McClenaghan, C.R. Barnett, F.P. O'Harte, P.R. Flatt, J Endocrinol. **151**, 349–357 (1996)
33. V.J. McKelvey-Martin, M.H. Green, P. Schmezer, B.L. Pool-Zobel, M.P. De Meo, A. Collins, Mutat. Res. 288, 47–63 (1993)
34. P.R. Flatt, C.J. Bailey, Diabetologia **20**, 573–577 (1981)
35. K. Grankvist, J. Sehlin, I.B. Taljedal, Acta Pharmacol. Toxicol. (Copenh.) **58**, 175–181 (1986)
36. H. Kaneto, J. Fujii, H.G. Seo, K. Suzuki, T. Matsuoka, M. Nakamura, H. Tatsumi, Y. Yamasaki, T. Kamada, N. Taniguchi, Diabetes **44**, 733–738 (1995)
37. E. Strandell, S. Sandler, J. Endocrinol. **153**, 81–86 (1997)
38. S.P. LeDoux, S.E. Woodley, N.J. Patton, G.L. Wilson, Diabetes **35**, 866–872 (1986)
39. Z. Ling, M. Van de Casteele, D.L. Eizirik, D.G. Pipeleers, Diabetes **49**, 340–345 (2000)
40. I. Flechner, K. Maruta, V. Burkart, K. Kawai, H. Kolb, U. Kiesel, Diabetes Res. **13**, 67–73 (1990)
41. N.S. Kwon, S.H. Lee, C.S. Choi, T. Kho, H.S. Lee, Faseb J. **8**, 529–533 (1994)
42. P.R. Flatt, S.K. Swanston Flatt, K.S. Tan, V. Marks, Gen. Pharmacol. **18**, 293–297 (1987)
43. S. Lortz, M. Tiedge, T. Nachtwey, A.E. Karlsen, J. Nerup, S. Lenzen, Diabetes **49**, 1123–1130 (2000)
44. M. Tiedge, H. Steffeck, M. Elsner, S. Lenzen, Diabetes **48**, 514–523 (1999)
45. N. Takasu, T. Asawa, I. Komiya, Y. Nagasawa, T. Yamada, J. Biol. Chem. **266**, 2112–2114 (1991)
46. T. Ishii, H. Iwahashi, R. Sugata, R. Kido, J. Biochem. (Tokyo) **104**, 610–615 (1988)
47. M. Poje, B. Rocic, M. Sikirica, I. Vickovic, M. Bruvo, J. Med. Chem. **26**, 861–864 (1983)
48. C.A. Delaney, D.L. Eizirik, Braz. J. Med. Biol. Res. **29**, 569–579 (1996)
49. Y. Uchigata, H. Yamamoto, A. Kawamura, H. Okamoto, J. Biol. Chem. **257**, 6084–6088 (1982)