biochemical pharmacology 76 (2008) 1748–1756

available at www.sciencedirect.com

journal homepage: www.elsevier.com/locate/biochempharm

Possible role of an ischemic preconditioning-like response mechanism in K_{ATP} channel opener-mediated protection against streptozotocin-induced suppression of rat pancreatic islet function

Stellan Sandler [a,*], Annika K. Andersson [a], Jenny Larsson [a], Natalia Makeeva [a], Therese Olsen [a], Per O.G. Arkhammar [c], John Bondo Hansen [c], F. Anders Karlsson [b], Nils Welsh [a]

[a] Department of Medical Cell Biology, Uppsala University, Uppsala, Sweden
[b] Department of Medical Sciences, Uppsala University, Uppsala, Sweden
[c] Novo Nordisk A/S, Måløv, Denmark

ARTICLE INFO

Article history:
Received 19 August 2008
Accepted 3 September 2008

Keywords:
Ischemic preconditioning
Mitochondria pancreatic islets
Potassium channel opener
Streptozotocin

ABSTRACT

Potassium channel openers (KCOs) decrease insulin secretion from β-cells. Some KCOs also protect against damage to β-cell function and type 1 diabetes in animal models. Previously we have found that the KCO NNC 55-0118 counteracted islet cell dysfunction, and this was associated with a lowering of the mitochondrial membrane potential (Δψ). Presently we aimed to explore whether inhibition of insulin secretion *per se* or rather inhibition of mitochondrial function correlates to counteraction of β-cell suppression. For this we used two novel KCOs (NNC 55-0321 and NNC 55-0462), which at certain concentrations have different actions regarding insulin secretion and the Δψ, with NNC 55-0321 being a potent inhibitor of Δψ and NNC 55-0462 being a potent inhibitor of insulin secretion. At 10 μM NNC 55-0321, but not with NNC 55-0462, the islet ATP content and ATP/ADP ratio was acutely decreased. This was accompanied by a complete protection against streptozotocin-induced suppression of islet insulin secretion using the former KCO. In cardiac research KCOs have been used to induce an ischemic preconditioning (IPC) response. In line with an IPC-like mechanism we found that NNC 55-0321 induced an initial free oxygen radical formation, PKC-ε isoform activation and a subsequent phosphorylation of the survival promoting factor Akt. Thus, KCOs may elicit mitochondrial events that resemble classical IPC seen in cardiomyocytes, and this could explain the enhanced islet cell function observed. KCOs with this property may be particularly interesting compounds to study as a rescue therapy during acute episodes of β-cell suppression/destruction.

© 2008 Elsevier Inc. All rights reserved.

1. Introduction

There are evidences that the vulnerability of the pancreatic β-cell to destructive mechanisms can vary, and that under certain conditions processes involved in cellular defence can be recruited [1,2]. Moreover, data suggest that reduced activity of the insulin-secreting cells is beneficial in newly onset diabetes in human [3–5] and that the activity of β-cell affects

* *Corresponding author*. Tel.: +46 18 4714430; fax: +46 18 4714059.
E-mail address: Stellan.Sandler@mcb.uu.se (S. Sandler).
0006-2952/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2008.09.004

the susceptibility to damage *in vitro* [6–8]. In this context the β-cell's insulin secretory activity has been believed to be decisive for how these cells will react to an insult. The use of potassium channel openers (KCOs) provides a tool for decreasing insulin secretion [9–11]. The drugs open the ATP sensitive potassium channel (K_{ATP} channel) in the plasma membrane leading to hyperpolarization of the plasma membrane and prevention of Ca^{2+}entry into the β-cell, the latter being crucial for insulin exocytosis.

We previously observed that pretreatment with diazoxide or the KCO, NNC 55-0118, selective for the Kir6.2/SUR1 K_{ATP} channel, has a protective effect against the toxic action of streptozotocin (STZ) on rat islets *in vitro* [12]. Furthermore, *in vivo* treatment with diazoxide or NNC 55-0118 was reported to maintain β-cell function in acutely diabetic BB rats [13]. Moreover, the KCO NN414 preserved insulin secretion of human islets exposed to elevated glucose concentrations *in vitro* [14], improved β-cell survival in BB rats [15] and prevented β-cell apoptosis of human islets [16]. In another study we demonstrated that KCOs, in particular NNC 55-0118, also prevented the toxic effects of alloxan and sodium nitroprusside [17]. In addition, IL-1β mediated suppression was reduced in the long-term presence of NNC 55-0118. In our studies the protection against the different noxious agents was provided by higher concentrations of KCOs than those needed for inhibition of insulin release [12,17]. The protection was correlated with a lowering of the mitochondrial membrane potential (Δψ).

In the present study we explored whether inhibition of insulin secretion *per se* or rather inhibition of mitochondrial function is correlated to protection against STZ. For this purpose we used two other KCOs, namely NNC 55-0321 and NNC 55-0462 ([10,11]; Fig. 1), which in certain concentration intervals have different actions regarding effects on insulin secretion and the Δψ. This also prompted us to explore if the protective action could be related to a so-called ischemic preconditioning (IPC)-like mechanism [18,19].

2. Materials and methods

2.1. *β-cell preparations and culture*

Pancreatic islets were isolated from adult male Sprague–Dawley rats (BK-Universal, Sollentuna, Sweden) by collagenase digestion and hand-picked. The islets were precultured free-floating in RPMI 1640 (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% FCS (v/v) (Sigma–Aldrich) and antibiotics for 5–6 days in air + 5% CO_2 at 37 °C. Medium was changed every second day. In some experiments mouse insulinoma βTC-6 cells ([20]; ATCC, Manassas, VA, USA) were used and maintained in Dulbecco's modified Eagle medium + 10% FCS and antibiotics. The passage number was 20–30 and the cells were used when 70% cell confluent. The use of rats was approved by the local Animal Ethics Committee (Tierp, Sweden).

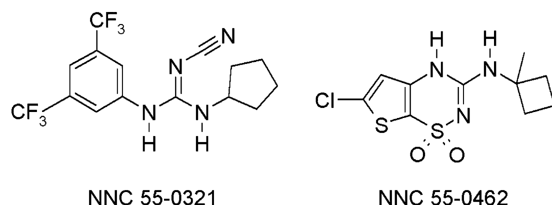

Fig. 1 – Chemical structures and correct chemical names of NNC 55-0321 and NNC 55-0462. NNC 55-0321 = N-cyano-N′-(3,5-bis(trifluoromethyl)phenyl)-N″-(cyclopentyl)guanidine and NNC 55-0462 = 6-chloro-3-(1-methylcyclobutyl)amino-4*H*-thieno[3,2-*e*]-1,2,4-thiadiazine 1,1-dioxide; cf. Refs. [9,10].

2.2. *Insulin secretion and dose–response experiments*

The potency of NNC 55-0321 and NNC 55-0462 to affect glucose-stimulated insulin secretion was examined in short-term incubations of islets at 16.7 mM glucose. Triplicates of five islets were transferred to 200 μl Krebs-Ringer bicarbonate buffer supplemented with 10 mM Hepes (KRBH; Sigma–Aldrich), 2 mg/ml BSA and 16.7 mM glucose in the absence or presence of NNC 55-0321 and NNC 55-0462 (0.01, 0.1, 1.0, 10 and 100 μM; Novo Nordisk Company, Copenhagen, Denmark) and incubated for 60 min at 37 °C. The insulin concentrations in the media were then determined with High-Range Rat Insulin ELISA (Mercodia, Uppsala, Sweden).

NNC 55-0321 and NNC 55-0462 were prepared as stock solutions in DMSO and diluted in KRBH 100–1000 times when added to the incubation media. In separate control experiments it was ascertained that the attained levels of DMSO did not affect islet insulin secretion (data not shown).

2.3. *STZ experiments*

The design of these experiments was adopted from [12]. Thus, cultured islets in groups of 40 were incubated in 1 ml KRBH (5.6 mM glucose +2 mg/ml BSA). Stock solutions of NNC 55-0321 and NNC 55-0462 were added to a final concentration of none, 1 or 10 μM for 30 min at 37 °C. Then STZ (Sigma) was dissolved in 0.9% saline, and 10 μl of saline or STZ solution was added for another 30 min (final STZ concentration 0.5 or 1.8 mM). The incubation was terminated by the addition of 2 ml cold KRBH and the islets transferred to culture dishes and allowed to recover for 24 h in medium RPMI 1640 + 10% FCS. Insulin secretion was then examined at 16.7 mM glucose, as described above. The data were expressed as % of the insulin secretory rate of control islets incubated in parallel, but not treated with STZ.

2.4. *Assessment of mitochondrial membrane potential*

To assess Δψ, 5,5′6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Molecular Probes Europe, Leiden, the Netherlands) fluorescence was measured [21]. Islets in groups of 300 were incubated in 4.5 ml RPMI 1640 + 0.5 ml FCS and 50 μl of JC-1 solution (4 μg/ml) for 20 min at 37 °C. Then islets were washed and dispersed into free cells by trypsin incubation (0.5%, w/v) in Ca^{2+}- and Mg^{2+}-free Hanks' medium (Sigma–Aldrich) ≈5 min at 37 °C. The dispersed cells were centrifuged and the cell pellet resuspended in culture medium and subsequently subdivided into five groups. After this NNC 55-0321 or NNC 55-0462 at different

concentrations (0, 0.1, 1.0, 10, 100 μM) were added to the islet cells for 30 min at 37 °C. Finally, the fluorescence was determined in a Becton Dickinson FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with respect to their FL_2 (590 nm) and FL_1 (530 nm) fluorescence, using an excitation wavelength at 488 nm. Data were analyzed using the CellQuest software (Becton Dickinson). The $\Delta\psi$ was expressed as a ratio FL2/FL1, and in each experiment the values of the cells exposed to NNC 55-0321 or NNC 55-0462 were calculated as a percentage of the corresponding control cells.

2.5. *Adenine nucleotide content*

Groups of 50 islets were incubated for 20 min at 37 °C in 400 μl RPMI 1640 (11.1 mM glucose) supplemented with 10% FCS and various concentrations the KCOs. The incubation was interrupted by removing 300 μl of the supernatant and 0.5 ml of 1 M cold perchloric acid was added to the 100 μl remaining incubation medium containing the islets. The solution was sonicated and then centrifuged for 5 min at 3500 rpm and the supernatant collected and stored frozen prior to measurement of adenine nucleotide content as described in detail elsewhere [22,23].

2.6. *PKC activation and Akt phosphorylation*

For analysis of PKC activation, βTC-6 cells were incubated (3×10^5) for 30 min in RPMI 1640 + 10% FCS without or with addition of 10 μM the KCOs, and for analysis of Akt phosphorylation 100 rat islets were incubated for 60 min as given above and then incubated for another 1, 3 or 6 h in the absence of KCOs. Following the different incubation periods the cells/islets were washed with cold PBS and used directly for preparation of membrane and cytosol fractions for analysis of PKC activation [24] or lysed in SDS-sample buffer for the determination of Akt phosphorylation. βTC-6 cell/islet proteins were separated on a 9% SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were incubated sequentially with rabbit anti-PKCε (C-15), rabbit anti-PKCδ (C-17), and rabbit anti-PKCζ (C-20) antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) followed by incubation with a horseradish peroxidase linked secondary antibody. Phosphorylation of Akt was assessed by immunoblotting using a Phospho-Akt (Ser473) antibody (Cell Signaling Technology, Beverly, MA, USA). Antibody binding was visualized by using the ECL immunoblotting detection system (Amersham Biosciences, Uppsala, Sweden). To normalize for differences in protein loading, the filters were stripped and rehybridized with rabbit anti-ERK1/2 antibodies (Cell Signaling Technology). Band intensities were quantified by densitometry and results were expressed as percent of the membrane/cytosol ratio of control cells (PKC) or percent of the P-Akt/total ERK ratio of control islet (P-Akt).

2.7. *iNOS gene expression*

βTC-6 cells and rat islets were cultured in the presence or absence of NNC 55-0321 and NNC 55-0462 (10 μM). One group of cells was exposed to human IL-1β (25 U/ml), an inducer of iNOS, as positive control. After 60 min the medium was changed to remove the KCOs, and the cells were then cultured 24, 36, 48 and 72 h before washed with PBS and lysed.

Total RNA was extracted using the RNeasy mini kit (Quiagen AG, Basel, Switzerland) and cDNA was synthesized by the cDNA Reverse Transcription System (Promega, Madison, WI, USA), and purified with the PCR-M Clean-Up System (Viogene, Sunnyvale, CA, USA). For the determination of iNOS mRNA expression, real-time PCR was performed, and for relative quantification, the house-keeping gene G6PDH was used. Primers for iNOS were from TIB Molbiol (Berlin, Germany) and for G6PDH from CyberGene (Huddinge, Sweden) and have been used previously [25]. The sequence of the primers used (Genebank accession number included) were for iNOS 5′-CAGCTGGGCTGTACAAACCTT-3′ (forward) and 5′-CATTGGAAGTGAAGCGTTTCG-3′ (reverse), M87039 and for G6PDH 5′-ATTGACCACTACCTGGGCAA-3′ (forward) and 5′-GAGATACACTTCAACACTTTGACCT-3′ (reverse), Z11911. Reactions were performed in a 10 μl reaction volume containing 1 μl cDNA, 2 μl RNase-free water, 5 μl SYBR Green Taq ReadyMix (Sigma–Aldrich) and 1 μM of the sense and antisense primers. The LightCycler (Roche Diagnostics GmBH, Mannheim, Germany) was used to perform real-time PCR and the size of the PCR products were verified on a 1.5% agarose gel. The crossing point values were compared between the samples and the reference G6PDH, rendering a ratio of iNOS/G6PDH mRNA levels.

2.8. *Generation of reactive oxygen species (ROS)*

Formation of ROS may precede PKC activation upon IPC [19]. We assessed production of ROS by flow cytometry, using the oxidation sensitive probe 5,6-carboxy-2′,7′-dichlorofluorescein diacetate (DCFH-DA; Acros Organics, New Jersey, NJ, USA). Once inside a cell DCFH-DA is cleaved to DCF which cannot penetrate the cell membrane. In the presence of hydrogen peroxide or hydroxyl radicals DCF will become fluorescent. Presently rat islets in groups of 100 were incubated in culture medium containing 10 μM DCFH-DA for 60 min at 37 °C. Isolated islets were then trypsinized (5 mg/ml) in 400 μl Ca^{2+}-free Hanks' salt solution for 4 min and terminated by the addition of 400 μl culture medium supplemented with 15 U of DNAase I. Fluorescence emission from DCF (green) was detected at a wavelength of 530 nm (time 0). Then vehicle alone (control), 10 μM of NNC 55-0321 or 10 μM NNC 55-0462 was added to aliquots of dispersed islet cells. DCF-fluorescence was determined by the FACS instrument at 2, 5, 15, 30 and 60 min after addition of the drugs. The technique described above has previously been successfully applied when studying insulin-producing cells exposed to cytokines [26].

2.9. *Statistical analysis*

Values are expressed as means ± S.E.M. and when applicable means were calculated from each duplicate or triplicate group and considered as one observation. Every observation relates to different islet donors when isolated islets were used. Student's paired or unpaired *t*-test or ANOVA for repeated measures including Bonferroni *t*-test, were used.

3. Results

The efficacy of NNC 55-0321 and NNC 55-0462 to inhibit insulin secretion at 16.7 mM glucose was examined in short-term incubations (Fig. 2). NNC 55-0321 did not affect insulin secretion at 0.01 or 0.1 μM, however, at 1.0 or 10 μM insulin secretion was inhibited by 43% and 57%, respectively. On the other hand, NNC 55-0462 decreased insulin release by about 60% at all tested concentrations.

Next, we investigated how NNC 55-0321 and NNC 55-0462 acutely influenced the islet cell mitochondrial membrane potential (Δψ) using the JC-1 probe (Fig. 3). At 0.1 or 1.0 μM neither NNC 55-0321 nor NNC 55-0462 had any effect on Δψ. However, NNC 55-0321 at 10 μM markedly inhibited Δψ (78%), while NNC 55-0462 had no effect. At 100 μM, Δψ was further suppressed by NNC 55-0321 (91%) and NNC 55-0462 caused some reduction in Δψ (49%), but not as marked as that caused by NNC 55-0321. Then we tested if the effects on Δψ were accompanied by changes in the islets adenine nucleotide content. Exposure of islets to NNC 55-0321 for 20 min caused a dose-dependent reduction of the islet ATP content (Fig. 4A). A significant reduction of ATP was first observed at 1.0 μM and this became further pronounced at 10 and 100 μM of NNC 55-0321 reaching a value about 20% of the control at the highest concentration. The decrease in ATP content coincided with an increase in the islet AMP content, whereas the ADP content remained unchanged. In similar experiments with NNC 55-0462 the ATP content became reduced by approximately 35% in the presence of 100 μM of the drug, while no effect was seen at 10 μM (Fig. 4B). The minor drop in the ATP content seen at 1.0 μM probably reflects a statistical type 1 error (Fig. 4B). The AMP content showed increases when there was a drop in the ATP content. The ADP content was not altered. The total adenine nucleotide content (pmol adenine nucleotides/50 islets) was also calculated and there was an about 30% and 13% decrease respectively as compared to the control islets at 100 μM of the KCOs (Control: 1383 ± 95.0; 100 μM NNC 55-0321: 952 ± 57.1 ($P < 0.05$ vs control); 100 μM NNC 55-0462: 1197 ± 133 ($P < 0.05$ vs control)). No significant effect on total adenine nucleotide content was observed at the other concentrations of the drugs tested (data not shown). The observed changes in ATP contents induced by the drugs were also reflected in the energy charges which is calculated as [1/2 ADP + ATP/AMP + ADP + ATP]. In the control islets the energy charge was 0.87 ± 0.10 (n = 5). Addition of NNC 55-0321 caused a gradual lowering to 0.39 ± 0.05 ($P < 0.001$) at 100 μM, while NNC 55-0462 induced a decrease to 0.59 ± 0.05 ($P < 0.01$) at the same concentration. The islet cell ATP/ADP ratio, which is of importance for regulation of glucose-induced insulin secretion, was significantly reduced by exposure to NNC 55-0321 at 10 and 100 μM, but not at 1.0 μM ($P < 0.07$ vs control) (Fig. 4C). NNC 55-0462 induced a decline in the ATP/ADP ratio only at 100 μM, despite that the compound suppressed insulin secretion at all tested concentrations (cf Fig. 2).

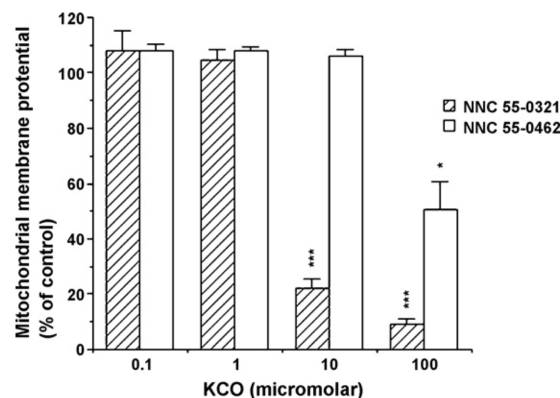

Fig. 3 – Mitochondrial membrane potential (Δψ), in rat pancreatic islets exposed at 37 °C on KRBH for 30 min without (control) or with KCOs at the concentrations indicated. The islets were then incubated with JC-1, trypsinized, and analyzed in a flow cytometer in which the fluorescence was measured at 588 nm (FL2) and 490 nm (FL1). The Δψ was expressed as a ratio FL2/FL1. Values are means ± S.E.M. for six experiments. * and * denote $P < 0.05$, and $P < 0.001$, respectively, compared to control islets not exposed to KCOs, using Student's paired *t*-test.**

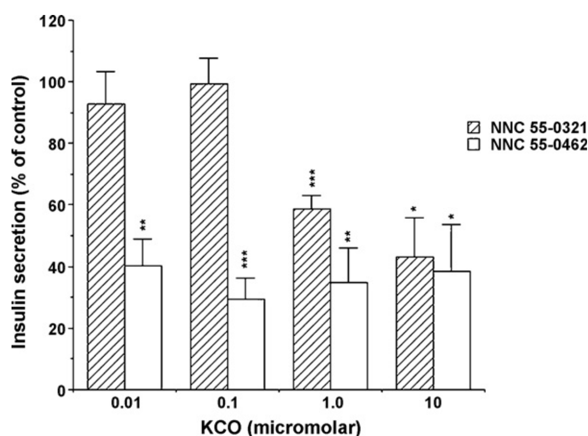

Fig. 2 – Acute effects of the two K_{ATP} channel openers (KCO) NNC 55-0321 and NNC 55-0462 on insulin secretion of rat pancreatic islets. The islets were incubated for 1 h in KRBH buffer containing 16.7 mM glucose without (control) or with KCOs at the concentrations indicated. Values are expressed as % of the insulin secretory rate of the controls islets not exposed to KCO. Values are means ± S.E.M. for five experiments and *, ** and * denote $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, vs control, using Student's paired *t*-test.**

In another series of experiments we aimed to investigate some different factors/pathways that have been implicated to be involved in different stages of IPC in studies mainly performed on cardiac tissue or cardiac cells [18,19]. Firstly, we examined if the KCOs induced iNOS mRNA, which could be a late manifestation after IPC (24–96 h) [18]. We did not observe iNOS gene expression in either isolated rat islets (n = 3) or βTC-6 cells (n = 3) after 24, 36, 48 or 72 h following a 1-h incubation with 10 μM of either NNC 55-0321 or NNC 55-0462. In parallel control experiments we exposed islets or cells to human IL-1β (25 U/ml), which caused a strong induction of iNOS mRNA after 6 h indicating that the transcript could be detected by the PCR method (data not shown). Moreover, generation of ROS during the first day has been connected to IPC. Using the

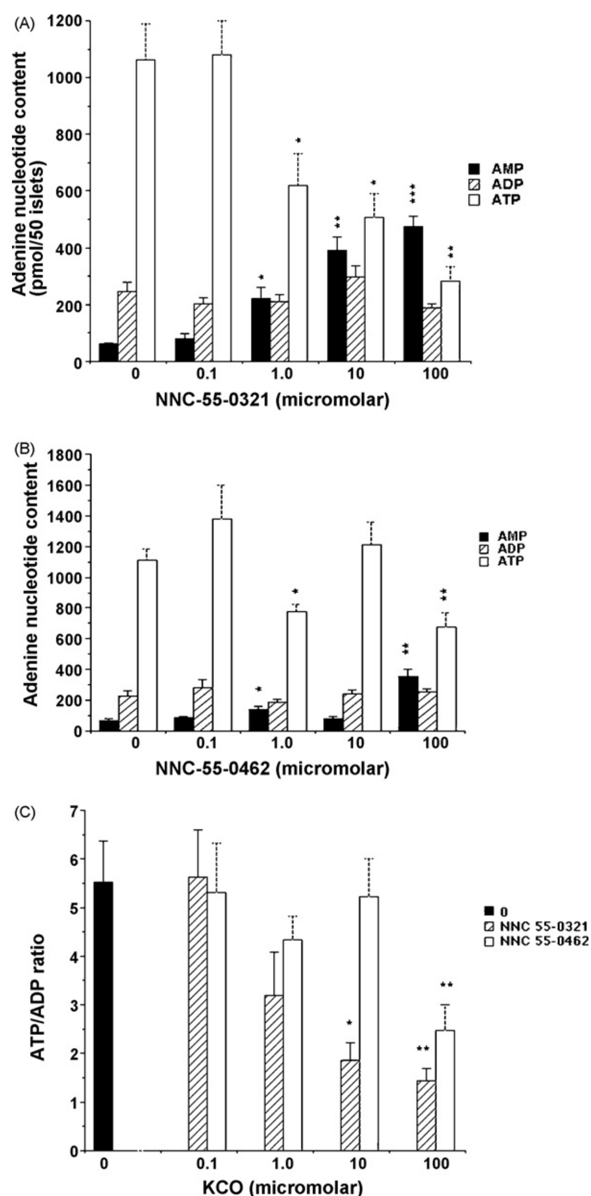

Fig. 4 – Acute effects of the KCOs NNC 55-0321 (A) and NNC 55-0462 (B) on islet contents of adenine nucleotides and on the islet ratio of ATP/ADP (C). Rat pancreatic islets were exposed at 37 °C in RPMI 1640 (11.1 mM glucose) supplemented with 10% FCS for 20 min without (0) or with NNC 55-0321. Then adenine nucleotides were extracted from the islets and measured by an HPLC method. Values are means ± S.E.M. for five experiments. *, ** and *** denote $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, compared to corresponding values of control islets (0) not exposed to KCO, using Student's paired *t*-test.

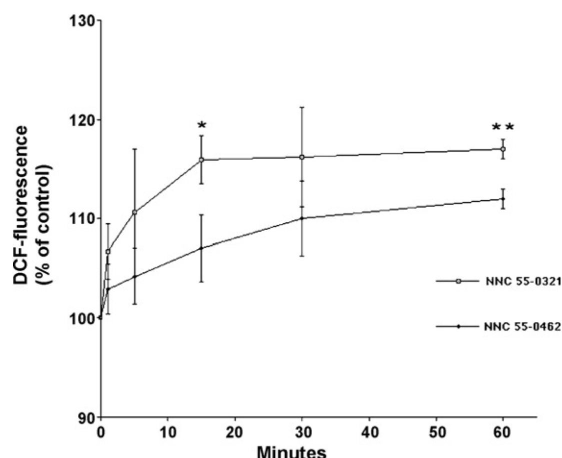

Fig. 5 – Effects of the KCOs NNC 55-0321 and NNC 55-0462 on formation of reactive oxygen species by rat islets using the oxidation sensitive probe 5,6-carboxy-2′,7′-dichlorofluorescein diacetate (DCFH-DA). Fluorescence emission from DCF (green) was detected at time 0. Then vehicle alone (control), 10 μM of NNC 55-0321 or 10 μM NNC 55-0462 was added to aliquots of dispersed islet cells, and fluorescence determined at 2, 5, 15, 30 and 60 min after addition of the drugs. Values are means ± S.E.M. for four experiments. * and ** denote $P < 0.05$ and $P < 0.01$, respectively, compared to corresponding fluorescence of control islets not exposed to KCO, using Student's paired *t*-test.

DCFH-DA probe we investigated if the KCOs affected ROS formation in rat islets (Fig. 5). During the 60 min observation period NNC 55-0321 induced an about 15% increase in DCF-fluorescence, indicating ROS formation, which was first seen after 15 min. NNC 55-0462 appeared also to increase DCF-fluorescence at a later stage, but this putative effect did not reach statistical significance. Activation of certain PKC isoforms (δ and ε) have been suggested to be a characteristic feature of IPC [27,28]. This was examined in insulin-producing βTC-6 cells after 30 min incubation with the KCOs (10 μM). We found PKCδ and PKCε activation after exposure to NNC 55-0321, but not to NNC 55-0462 (Table 1). The phorbol myristic aldehyde (PMA) was used as a positive control. We also determined PKCζ since this isoform is not supposed to be

Table 1 – Effects of KCOs on the distribution of PKC isoforms in βTC-6 cells

Treatment	PKC isoform (membrane/cytosol)		
	PKCδ	PKCζ	PKCε
0	0.63 ± 0.06	0.79 ± 0.17	0.19 ± 0.02
PMA (100 nM)	1.44 ± 0.12**	0.71 ± 0.11	0.82 ± 0.18*
NNC 55-0321 (10 μM)	1.18 ± 0.06**	0.65 ± 0.25	0.94 ± 0.07*
NNC 55-0462 (10 μM)	0.97 ± 0.11	0.58 ± 0.07	0.69 ± 0.26

Cells (3×10^5) were incubated for 30 min with the additions given. Membrane and cytosol proteins, were separated and transferred to a nitrocellulose membrane. The membranes were sequentially incubated with rabbit anti-PKCε, -PKCδ, and -PKCζ antibodies, followed by incubation with a secondary antibody. Antibody binding was visualized by using the ECL immunoblotting detection system. To normalize for differences in protein loading, the filters were stripped and rehybridized with rabbit anti-ERK1/2 antibodies. Band intensities were quantified and results expressed as ratio between the membrane/cytosol. Values are means ± S.E.M. from 3 experiments. * and ** denote $P < 0.05$ and $P < 0.01$, vs control (0).

activated by either PMA or IPC, and there was no translocation observed of PKCζ. In investigations of cardioprotection it has been reported that PKCε can phosphorylate the anti-apoptotic protein Akt [28]. In rat islets we found an increased phosphorylation of Akt 1 h after incubation with NNC 55-0321, while after 3 and 6 h there was not a statistical difference vs the non-KCO exposed control (Fig. 6). Incubation with NNC 55-0462 did not show any change at the timepoints tested.

Previously we observed that the presence of another KCO, NNC 55-118, which both inhibited insulin secretion and reduced Δψ at the concentration used, protected against suppression of insulin secretion by β-cell toxins STZ and alloxan [12,17]. We therefore tested if NNC 55-0321 and NNC 55-0462 could have a similar protective effect. In these experiments we employed STZ and selected to use 1 and 10 μM of the drugs, since we had found that both KCOs inhibited glucose-stimulated insulin secretion at these concentrations (Fig. 2). On the other hand, NNC 55-0321 markedly decreased Δψ and the ATP content, whereas NNC 55-0462 had little or none such effects at 1 or 10 μM (Figs. 3 and 4). The experimental design was that the islets were preincubated for 30 min with the KCOs then STZ was added for another 30 min,

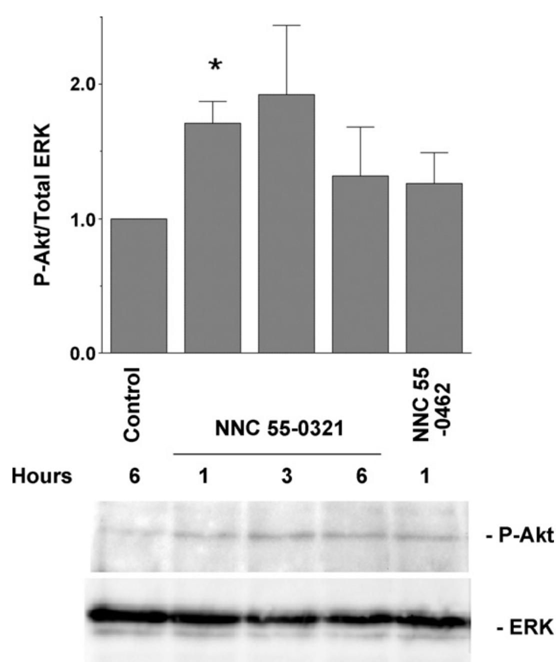

Fig. 6 – Effects of the KCOs NNC 55-0321 and NNC 55-0462 on phosphorylation of Akt. Rat islets in groups of 100 were incubated for 60 min with 10 μM of either NNC 55-0321 or NNC 55-0642. The islets were then transferred to culture medium with no KCO supplementation and incubated further for 1, 3 or 6 h. The islets were washed in cold PBS and solubilized in SDS-sample buffer for PAGE and immunoblotting for phospho-Akt (Ser473) and total ERK. Total ERK was used as loading and blotting control. The upper panel shows densitometric scanning results which are presented as means ± S.E.M. for 4–6 observation. * denotes $p < 0.05$ using Students paired t-test. The lower panel shows a photograph of one representative experiment.

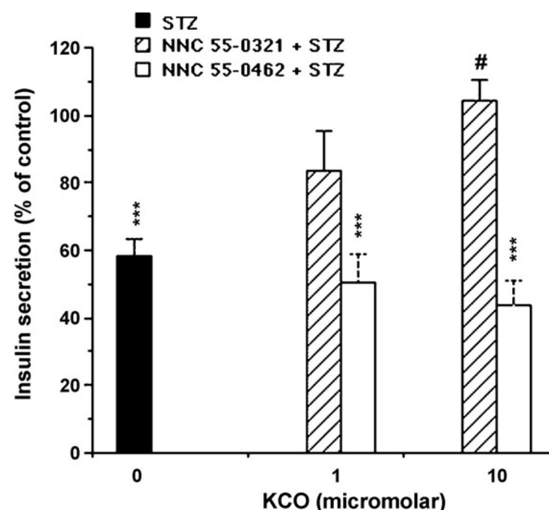

Fig. 7 – Effects of the two KCOs NNC 55-0321 and NNC 55-0462 on streptozotocin (STZ)-induced inhibition of insulin secretion of rat pancreatic islets. Islets were incubated 30 min at 37 °C with addition of saline (0) or KCOs as indicated. Then STZ (0.5 mmol/l) was added to the islets for another 30 min, allowed to recover for 24 h in culture medium. Insulin secretion was subsequently measured 16.7 mM glucose in triplicate groups of five islets. The data were expressed as % of the insulin secretory rate of control islets incubated in parallel, but not treated with STZ or KCOs. Values are means ± S.E.M. for seven experiments. ** and * denote $P < 0.01$ and $P < 0.001$, respectively, compared to values of control islets not exposed to KCO (0) and # denotes $P < 0.05$ compared to islets exposed to 10 μmol/l NNC 55-0462 + STZ, using ANOVA for repeated measures including Bonferroni t-test.**

the islets washed and transferred to normal culture medium and the glucose-stimulated insulin secretion examined after 24 h. It was found that treatment of control islets with 0.5 mM STZ lead to a more than 40% inhibition of the insulin release (Fig. 7). Addition of NNC 55-0462 at 1 or 10 μM did not influence the STZ-induced suppression of insulin release. However, NNC 55-0321 at 10 μM fully protected against STZ inhibition, and also 1 μmol/l of the drug counteracted this effect, although this was not significantly different from the group of islets treated with STZ alone ($P < 0.064$). Furthermore, we performed experiments in which 10 μM NNC 55-0321 was withdrawn during the 30 min incubation with STZ after the 30 min preceding exposure to the KCO. Then after culture for 24 h in RPMI 1640 + 10% FCS the islets were first incubated at low glucose (1.7 mM) and during a second hour at high glucose (16.7 mM). Also with this experimental design NNC 55-0321 prevented STZ-induced suppression of glucose-stimulated insulin release (relative stimulation of insulin release 16.7/1.7 mM glucose ($n = 3$) Control: 6.5 ± 2.3; STZ alone: 1.4 ± 0.1; NNC 55-0321 alone: 7.3 ± 0.7; STZ + NNC 55-0321: 5.6 ± 0.5 ($P < 0.001$ vs STZ alone, unpaired t-test)). This excludes that NNC 55-0321 would exert its effect by a direct interaction with the STZ molecule or prevent the uptake of STZ.

4. Discussion

Previous characterization of the KCOs has shown that NNC 55-0321 ([11]; compound 10) could repolarize glucose-induced depolarization of cellular membranes of insulin-producing βTC-3 cells (EC_{50} 4.7 ± 0.5 μM) and inhibit glucose-stimulated insulin release from βTC-6 cells (IC_{50} 5.5 ± 1.9 μM), and the corresponding data for NNC 55-0462 ([10]; compound 54) were (EC_{50} 0.014 ± 0.006 μM) and (IC_{50} 0.02 ± 0.004 μM). Also patch clamp recordings of K^+ currents show that the drugs have KCO properties ([11]; Gaisano et al. data not shown). In the present investigation of rat pancreatic islets NNC 55-0462 markedly inhibited insulin release already at 0.01 μM, whereas a 100–1000-fold higher concentration of NNC 55-0321 was required to obtain a similar effect (Fig. 2). On the other hand, using NNC 55-0462 the Δψ was found to be suppressed at more than a 10-fold higher concentration compared to that required for NNC 55-0321 (Fig. 3). In addition, a pronounced and consistent decline in islet ATP content and energy charge was observed at a 10–100-fold lower concentration of NNC 55-0321 compared to NNC 55-0462 (Figs. 4A–C). Together with the previous observations [10,11] this indicates that NNC 55-0462 affects plasma membrane K_{ATP} channels and insulin release with a much higher potency than NNC 55-0321. However, NNC 55-0321 affects mitochondrial function at a markedly lower concentration than NNC 55-0462.

When we examined a putative protective effect against a classical β-cell cytotoxin we selected STZ and the KCOs were studied at 1 μM and 10 μM. It was found that NNC 55-0321 exerted a protective action against STZ-induced inhibition of insulin secretion, while NNC 55-0462 was ineffective. From this it becomes apparent that it is a suppression of mitochondrial function rather than inhibition of insulin secretion *per se* that is associated with a protection against STZ cytotoxicity. Mitochondrial function and efficient ATP generation is crucial for the β-cell both for its general survival and for an appropriate production and release of insulin in response to nutrient secretagogues [29,30]. We have earlier favoured the view that a high-energy state makes the β-cell better equipped to handle an aggression by noxious agents and this would also facilitate cellular defence processes [1]. Against this background it seems paradoxical that a dissipation of mitochondrial energy production, as presently seen with NNC 55-0321, would counteract islet cell damage induced by STZ. We interpret this that if the islet cell ATP supply is transiently suppressed by a KCO, signals are generated which confer protection against STZ-induced β-cell damage. Provided that the mitochondrial suppression is reversed after withdrawal of the KCO, then the β-cell function will become normal at a later timepoint despite the exposure to the noxious compound.

In other tissues, for example, the heart, muscle and CNS, protective actions by KCOs against hypoxia and various toxins

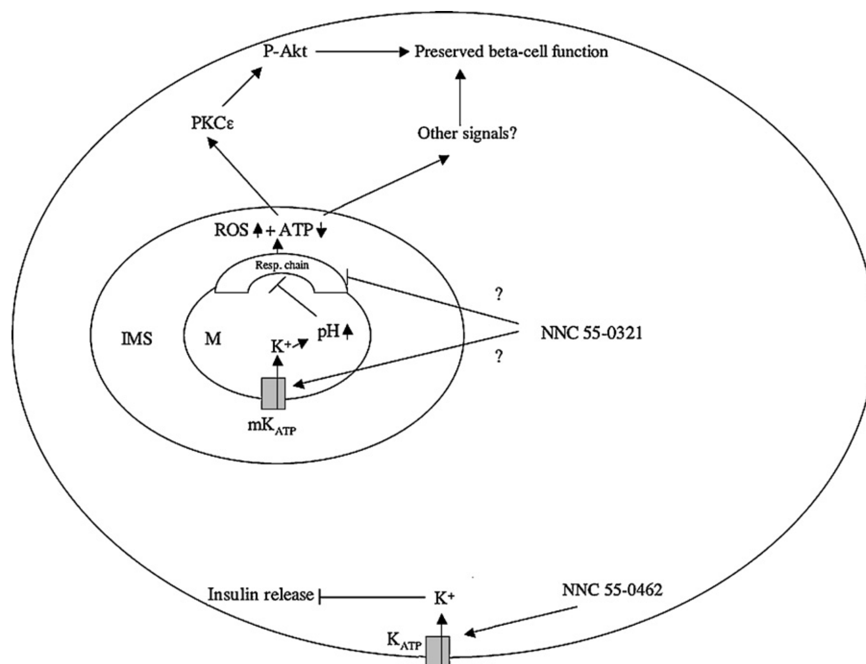

Fig. 8 – Proposed mechanism for NNC 55-0321-mediated protection against β-cell suppression. The KCO NNC 55-0321 acutely down-regulates mitochondrial function as assessed by Δψ and energy charge determinations. The lowered respiratory chain activity is accompanied by increased ROS production, PKCε activation and subsequently a phosphorylation of the survival promoting kinase Akt. The inhibition of mitochondrial function by NNC 55-0321 may be caused by opening of a putative mitochondrial potassium channel (mK_{ATP}), which promotes K^+ entry from the intermembrane space (IMS) into the mitochondrial matrix (M), thereby increasing pH and inhibiting the respiratory chain (I). Alternatively, the NNC 55-0321 can directly inhibit mitochondrial respiration independently of the presence of and conductance in an mK_{ATP} (II). The KCO NNC 55-0462 primarily acts on the plasma membrane bound K_{ATP} channel of the β-cell and effectively causes inhibition of insulin secretion by preventing depolarization of the plasma membrane, but this does not provide protection against β-cell damage.

have been reported [31–34] a phenomenon that has been designated as IPC [35,36]. K_{ATP} channels have thus been suggested to be present in the sarcoplasmic reticulum [37] and in mitochondria [38] and it has been postulated that K_{ATP} channels of mitochondria are mediating the IPC [32]. In contrast to the K_{ATP} channel present in the plasma membrane of the β-cell [39,40], mitochondrial K_{ATP} channels are yet not cloned by molecular biology techniques and their actual existence are deduced essentially from pharmacological experiments [32,38]. Nevertheless, it has been proposed that opening of mitochondrial K_{ATP} channels would leads to ROS generation due to an increase in pH and to inhibition of respiratory chain complexes in the inner membrane of the mitochondria [41,42]. Alternatively, it may be that KCOs inhibit the respiratory chain and stimulate ROS production directly since it has also recently been suggested the KCO diazoxide can induce respiratory chain inhibition independently of the conductance in the putative mitochondrial K_{ATP} channels [43]. Our previous [12,17] and present findings are compatible with the view that mitochondrial K_{ATP} channels may be present in rat islet cells. In line with findings in cardiac cells we found that NNC 55-0321 acutely increased islet cell generation of ROS, which takes place in IPC [27,44]. Furthermore, as also seen in IPC [38,44], we presently observed an acute activation of PKC isoforms δ and ε by NNC 55-0321, but not by NNC 55-0462. Subsequently we found that exposure to NNC 55-0321 caused phosphorylation of the anti-apoptotic protein Akt (Fig. 7), as reported to occur in cardioprotection via catalysis by PKCε [28] as well in survival of glioma cells [45] and breast cancer cells [46]. We did not find any increase in iNOS gene expression when both βTC-6 cells and rat islets were followed for up to 72 h after treatment with the KCOs. Induction of iNOS is considered a late protective IPC event [18], but our findings do not support the presence of such a mechanism in islet cells. Hence iNOS induction in rodent islets is likely to result in impaired β-cell function [47]. Based on our findings herein we propose a model how certain KCOs (e.g., NNC 55-0321) can promote preservation of β-cell function (Fig. 8). It is suggested that NNC 55-0321 via an action affecting mitochondrial energy generation elicits signal pathways which may preserve β-cell function during acute damage. The signals involved have features resembling IPC [18,19]. Another KCO, NNC 55-0462 primarily acts on the plasma membrane located K_{ATP} channel of the β-cell [39] causing inhibition of insulin secretion by preventing depolarization of the plasma membrane.

In conclusion we have found that exposure of rat pancreatic islets to a drug that transiently decreases the islet ATP content, mediates protection against damage induced by STZ. This suggests that specific KCOs, via down-regulation of mitochondrial energy production and induction of anti-apoptotic and/or other signals, can interrupt the processes leading to suppression of β-cells. Thus, KCOs may be interesting drugs to study as a rescue therapy during an acute episode of massive beta cell damage.

Acknowledgements

We are highly indebted to Margareta Ericson, Åke Ericson and Ing-Britt Hallgren for excellent technical assistance. This study was supported by grants from the Swedish Research Council, the Novo Nordic Fund, the European Foundation for the Study of Diabetes, the Swedish Diabetes Association and the Family Ernfors Fund.

REFERENCES

[1] Eizirik DL, Sandler S, Palmer JP. Repair of pancreatic β-cells. A relevant phenomenon in early IDDM in different glucose concentrations modifies B cell sensitivity to streptozotocin. Diabetes 1993;42:1383–91.

[2] Pipeleers D, Hoorens A, Marichal-Pipeleers M, van de Casteele M, Bouwens L, Ling Z. Role of pancreatic β-cells in the process of β-cell death. Diabetes 2001;50(Suppl. 1): S52–7.

[3] Björk E, Berne C, Kämpe O, Wibell L, Oskarsson P, Karlsson FA. Diazoxide treatment at onset preserves residual insulin secretion in adults with autoimmune diabetes. Diabetes 1996;45:1427–30.

[4] Örtqvist E, Björk E, Wallensteen M, Ludvigsson J, Åman J, Johansson C, et al. Temporary preservation of β-cell function by diazoxide treatment in childhood type 1 diabetes. Diabetes Care 2004;27:2191–7.

[5] Vondra K, Voborska M, Kvapil M, Weber P, Dvorakova H, Stanicka S, et al. Somatostatin: beneficial effects on remission in young adult patients with newly diagnosed diabetes mellitus type 1. Physiol Res 2004;53:115–7.

[6] Eizirik DL, Strandell E, Sandler S. Culture of mouse pancreatic islets in different glucose concentrations modifies B cell sensitivity to streptozotocin. Diabetologia 1988;31:168–74.

[7] Palmer JP, Helqvist S, Spinas GA, Mølvig J, Mandrup-Poulsen T, Andersen HU, et al. Interaction of β-cell activity and IL-1 concentration and exposure time in isolated rat islets of Langerhans. Diabetes 1989;38:1211–6.

[8] Spinas GA, Palmer JP, Mandrup-Poulsen T, Andersen H, Nielsen JH, Nerup J. The bimodal effect of interleukin 1 on rat pancreatic β-cells - stimulation followed by inhibition - depends upon dose, duration, and ambient glucose concentration. Acta Endocrinol (Copenh) 1988;119:307–11.

[9] Mannhold R. K_{ATP} channel openers: structure-activity relationships and therapeutic potential. Med Res Rev 2004;24:213–66.

[10] Nielsen FE, Bodvarsdottir TB, Worsaae A, MacKay P, Stidsen CE, Boonen HC, et al. 6-Chloro-3-alkylamino-4*H*-thieno[3,2-*e*]-1,2,4-thiazidine 1,1-dioxide derivatives potently and selectively activate ATP sensitive potassium channels of pancreatic β-cells. J Med Chem 2002;45:4171–87.

[11] Tagmose TM, Schou SC, Mogensen JP, Nielsen FE, Arkhammar PO, Wahl P, et al. Arylcyanoguanidines as activators of Kir6.2/SUR1K_{ATP} channels and inhibitors of insulin release. J Med Chem 2004;47:3202–11.

[12] Kullin M, Li ZC, Hansen JB, Björk E, Sandler S, Karlsson FA. K_{ATP} channel openers protect rat islets against the toxic effect of streptozotocin. Diabetes 2000;49:1131–6.

[13] Skak K, Gotfredsen CF, Lundsgaard D, Hansen JB, Sturis J, Markholst H. Improved β-cell survival and reduced insulitis in a type 1 diabetic rat model after treatment with a β-cell-selective K_{ATP} channel opener. Diabetes 2004;53:1089–95.

[14] Ritzel RA, Hansen JB, Veldhuis JD, Butler PC. Induction of β-cell rest by a Kir6. 2/SUR1-selective K_{ATP} channel opener preserves β-cell insulin stores and insulin secretion in human islets cultured at high (11 mM) glucose. J Clin Endocrinol Metab 2004;89:795–805.

[15] Rasmussen SB, Sørensen TS, Hansen JB, Mandrup-Poulsen T, Hornum L, Markholst H. Functional rest through

intensive treatment with insulin and potassium channel openers preserves residual β-cell function and mass in acutely diabetic BB rats. Horm Metab Res 2000;32:294–300.
[16] Maedler K, Størling J, Sturis J, Zuellig RA, Spinas GA, Arkhammar PO, et al. Glucose and interleukin-1β-induced β-cell apoptosis requires Ca^{2+} influx and extracellular signal-regulated kinase (ERK)1/2 activation and is prevented by a sulphonylurea receptor 1/inwardly rectifying K^+ channel 6.2 (SUR/Kir6.2) selective potassium channel opener in human islets. Diabetes 2004;53:1706–13.
[17] Kullin M, Li Z, Hansen JB, Welsh N, Karlsson FA, Sandler S. Protection of rat pancreatic islets by potassium channel openers against alloxan, sodium nitroprusside and interleukin-1β mediated suppression—possible involvement of the mitochondrial membrane potential. Diabetologia 2003;46:80–8.
[18] Bolli R. The late phase of preconditioning. Circ Res 2000;87:972–83.
[19] Murphy E. Primary and secondary signalling pathways in early preconditioning that converge on the mitochondria to produce cardioprotection. Circ Res 2004;94:7–16.
[20] Knaack D, Fiore DM, Surana M. Clonal insulinoma cell line that stably maintains correct glucose responsiveness. Diabetes 1994;43:1413–7.
[21] Salvioli S, Ardizzoni A, Franceschi C, Cossarizza A. JC-1, but not DiOC6(3) or rhodamine123, is a reliable fluorescent probe to assess Δψ changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. FEBS Lett 1997;411:77–82.
[22] Ericson Å, Niklasson F, de Verdier C-H. A systematic study of nucleotide analysis of human erythrocytes using an anionic exchanger and HPLC. Clin Chim Acta 1983;127:47–59.
[23] Sellevold OFM, Jynge P, Aarstad K. High performance liquid chromatography of creatine compounds and adenine nucleotides in myocardial tissue. J Mol Cell Cardiol 1986;18:517–27.
[24] Alcazar O, Qiu-yue Z, Gine E, Tamarit-Rodriguez J. Stimulation of islet protein kinase C translocation by palmitate requires metabolism of the fatty acid. Diabetes 1997;46:1153–8.
[25] Andersson AK, Thorvaldson L, Carlsson C, Sandler S. Cytokine-induced PGE_2 formation is reduced from iNOS deficient murine islets. Mol Cell Endocrinol 2004;220:21–9.
[26] Barbu A, Welsh N, Saldeen J. Cytokine-induced apoptosis and necrosis are preceded by disruption of the mitochondrial membrane potential (Δψ) in pancreatic RINm5F cells: prevention by Bcl-2. Mol Cell Endocrinol 2002;190:75–82.
[27] Raval AP, Dave KR, Mochly-Rosen D, Sick TJ, Pérez-Pinzón MA. εPKC is required for the induction of tolerance by ischemic and NMDA-mediated preconditioning in the organotypic hippocampal slice. J Neurosci 2003;23:384–91.
[28] Zhang J, Baines CP, Zong C, Cardwell EM, Wang G, Vondriska TM, et al. Functional proteomic analysis of a three-tier PKCε-Akt-eNOS signaling module in cardiac protection. Am J Physiol Heart Circ Physiol 2005;288:H954–61.
[29] Malaisse WJ. Glucose-sensing by the pancreatic B-cell: the mitochondrial part. Int J Biochem 1992;24:693–701.
[30] Meglasson MD, Matschinsky FM. Pancreatic islet glucose metabolism and regulation of insulin secretion. Diabetes Metab Rev 1986;2:163–214.
[31] Cole WC, McPherson CD, Sontag D. ATP-regulated K^+ channels protect the myocardium against ischemia/reperfusion damage. Circ Res 1991;69:571–81.
[32] Garlid KD, Paucek P, Yarov-Yarovoy V, Murray HN, Darbenzio RB, D'Alonzo AJ, et al. Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K^+ channels. Possible mechanism of cardioprotection. Circ Res 1997;81:1072–82.
[33] Goodman Y, Mattson MP. K^+ channel openers protect hippocampal neurons against oxidative injury and amyloid β-peptide toxicity. Brain Res 1996;706:328–32.
[34] Patel MN, Yim GK, Isom GE. Potentiation of cyanide neurotoxicity by blockade of ATP-sensitive potassium channels. Brain Res 1992;593:114–6.
[35] Liu GC, Vasquez JA, Gallagher KP, Lucchesi BR. Myocardial protection with preconditioning. Circulation 1990;82:609–19.
[36] Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 2004;74:1124–36.
[37] Yellon D. ATP-dependent potassium channel openers and preconditioning. Cardiovasc Res 1993;27:1882–3.
[38] Inoue I, Nagase H, Kishi K, Higuti T. ATP-sensitive K^+ channels in mitochondrial inner membrane. Nature 1991;352:244–7.
[39] Aguilar-Bryan L, Nichols CG, Wechsler S, Clement IV JP, Boyd III AE, Gonzales G, et al. Cloning of the β-cell high-affinity receptor: a regulator of insulin secretion. Science 1995;268:423–6.
[40] Inagaki N, Gonoi T, Clement IV JP, Namba N, Inazawa J, Gonzales G, et al. Reconstitution of K_{ATP}: an inward rectifier subunit plus the sulfonylurea receptor. Science 1995;270:1166–70.
[41] Andrukhiv A, Costa AD, West IC, Garlid K. Opening mitoK_{ATP} increases superoxide generation from complex I of the electron transport chain. Am J Physiol Heart Circ Physiol 2006;291:H2067–74.
[42] Kukreja RC. Mechanism of reactive oxygen species generation after opening of mitochondrial K_{ATP} channels. Am J Physiol Heart Circ Physiol 2006;291:H2041–3.
[43] Minners J, Lacerda L, Yellon DM, Opie LH, McLeod CJ, Sack MN. Diazoxide-induced respiratory inhibition—a putative mitochondrial K_{ATP} channel independent pharmacological preconditioning. Mol Cell Biochem 2007;294:11–8.
[44] Soujorn MC, Mochly-Rosen D. Peptide modulators of protein–protein interaction in intracellular signaling. Nat Biotechnol 1998;16:19–24.
[45] Okhrimenko H, Lu W, Xiang C, Hamburger N, Kazimirsky G, Brodie C. Protein kinase C-ε regulates the apoptosis and survival of glioma cells. Cancer Res 2005;65:7301–9.
[46] Lu D, Huang J, Basu A. Protein kinase Cε activates protein kinase B/Akt via DNA-PK to protect against tumor necrosis factor-α-induced cell death. J Biol Chem 2006;281:22799–807.
[47] Eizirik DL, Flodström M, Karlsen AE, Welsh N. The harmony of the spheres: inducible nitric oxide synthase and related genes in pancreatic beta cells. Diabetologia 1996;39:875–90.