Short-term intermittent exposure to diazoxide improves functional performance of β-cells in a high-glucose environment

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Yoshikawa, Hiroyasu, Zuheng Ma, Anneli Björklund, and Valdemar Grill. Short-term intermittent exposure to diazoxide improves functional performance of β-cells in a high-glucose environment. Am J Physiol Endocrinol Metab 287: E1202–E1208, 2004.—Prolonged periods of “β-cell rest” exert beneficial effects on insulin secretion from pancreatic islets subjected to a high-glucose environment. Here, we tested for effects of short-term intermittent rest achieved by diazoxide. Rat islets were cultured for 48 h with 27 mmol/l glucose alone, with diazoxide present for 2 h every 12 h or with continuous 48-h presence of diazoxide. Both protocols with diazoxide enhanced the postculture insulin response to 27 mmol/l glucose, to 200 μmol/l tolbutamide, and to 20 mmol/l KCl. Intermittent diazoxide did not affect β-cell insulin content and enhanced only KATP-dependent secretion, whereas continuous diazoxide increased β-cell insulin contents and enhanced both KATP-dependent and -independent secretory effects of glucose. Intermittent and continuous diazoxide alike increased postculture ATP-to-ADP ratios, failed to affect [%14C]glucose oxidation, but decreased oxidation of [%14C]oleate.

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1640 medium containing 11 mmol/l glucose supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in a humidified (5% CO2-95% air) atmosphere.

**Experimental design.** After the overnight culture, islets were further cultured for 48 h by different protocols, most of which employed various times of exposure to diazoxide (Fig. 1).

**Insulin.** After the 48-h culture, final batch-type incubations were carried out as previously described (13). Briefly, to measure insulin release, islets were incubated in Krebs-Ringer bicarbonate (KRB) medium (22), which was supplemented with 10 mmol/l HEPES, 0.2% BSA, and 3.3 mmol/l glucose. Triplicates of three isolated islets for each condition were incubated for 60 min in 300 µl of KRB with additions dictated by the experimental protocol. Aliquots of the incubation medium were removed for assay of insulin concentration. Islets that had been exposed to 3.3 mmol/l glucose during final incubations were retrieved and transferred into 200 µl of acid-ethanol (0.18 mol/l HCl in 95% ethanol) for later sonication and determination of insulin content.

Insulin was measured by RIA using rat insulin as standard, moniodinated porcine insulin as tracer, and antibodies against porcine insulin. Antibody-bound insulin was separated from free insulin using Dextran 170-coated charcoal (6).

**ATP and ADP contents.** After culture, batches of 10 islets were put into Eppendorf tubes containing 40 µl of NaOH solution (0.04 mol/l NaOH, 2 mmol/l EDTA) and stored at −80°C. Before assay, 60 µl of lysis reagent were added to islets, the lystate was passed through a 23-gauge needle, and vortexed ADP was converted to ATP with 2.3 U/ml pyruvate kinase and 1.5 mmol/l phosphoenolpyruvate for 15 min at room temperature. ATP was assessed by luminometric determination of the luciferin-luciferase reaction by use of a commercially available assay (Boehringer Mannheim).

**Apoptosis.** Islets cell death/survival was assessed by ELISA by the Cell Death Plus assay (Roche). Aliquots of 10 islets of comparable size were incubated for 30 min with a lysis buffer at room temperature and then centrifuged at 200 g for 10 min at 4°C. Aliquots of the supernatant (20 µl) were placed into microtiter plate wells coated with streptavidin. A total of 80 µl of a solution containing 2,2’-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (the substrate for peroxidase) were added. At the end of a 15-min incubation, absorbance of sample was read spectrophotometrically at 405 nm.

**RT-PCR.** Total RNA was extracted from 100 islets with a High Pure RNA isolation kit (Roche Diagnostics). Total RNA was transcribed by random priming using reverse transcripts (RT; first-strand DNA synthesis) according to instructions from the manufacturer (Invitrogen, Oslo, Norway). One microliter of the RT reaction mix was amplified with primers specific for uncoupling protein-2 (UCP2) (11) (Kir6.2) (8), sulfonylurea receptor 1 (SUR1) (12), voltage-dependent Ca2+/channel-α1 (VDCCα1) (8), voltage-dependent K+ 2.1 channel (Kv2.1) (24), and β-actin (19) in a total volume of 50 µl. Details on primers and constructs are given in Table 1. Linearity of the PCR reaction was tested by amplification. The samples were amplified using the following conditions: 92°C for 30 s, 55–59°C for 30 s, and 68°C for 1 min. Aliquots (10 µl) of the PCR mixture were run on 1% agarose gels followed by staining with ethidium bromide. Signals were quantified by scanning densitometry using molecular Analyst software (Bio-Rad, Hercules, CA).

**Glucose and oleate oxidation.** The production of 14CO2 from D-[U-14C]glucose was measured basically as previously described (9). After culture, islets were preincubated for 30 min in KRB at 3.3 mmol/l glucose. Duplicates of 10 islets each were then placed in 1-ml glass vials containing 100 µl of KRB medium together with 0.5 µCi of D-[U-14C]glucose plus nonradioactive glucose to a final concentration of either 3.3 or 27 mmol/l. The glass vials were placed in 20-ml scintillation bottles that were gassed with O2-CO2 (95:5) and capped airtight with rubber membranes. The bottles were shaken continuously for 120 min at 37°C in a water bath. Islet metabolism was stopped by an injection of 250 µl of 0.1 mol/l HCl into the glass vials followed by injection of 250 µl of hyamine hydroxide into the scintillation bottles. These were sealed and left overnight at room temperature to absorb 14CO2 into hyamine. Blank incubations were treated identically. Oxidation of glucose was calculated as picomoles of glucose per 10 islets per 2 h.

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**Fig. 1.** Experimental design, illustrating and defining the various protocols.

**Table 1**

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>HG</td>
<td>27mM glucose</td>
</tr>
<tr>
<td>HD48/48</td>
<td>27mM glucose + diazoxide</td>
</tr>
<tr>
<td>HD64/48</td>
<td>27mM glucose diazoxide 27mM glucose diazoxide 27mM glucose diazoxide 27mM glucose diazoxide</td>
</tr>
<tr>
<td>HD6/48</td>
<td>27mM glucose diazoxide 27mM glucose diazoxide 27mM glucose diazoxide 27mM glucose</td>
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<td>HD2/48</td>
<td>27mM glucose diazoxide</td>
</tr>
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<td>HL4/48</td>
<td>27mM glucose 5.5mM glucose 27mM glucose 5.5mM glucose 27mM glucose 5.5mM glucose 27mM glucose 5.5mM glucose</td>
</tr>
<tr>
<td>day 1</td>
<td>10 h 2 h 10 h 2 h</td>
</tr>
<tr>
<td>day 2</td>
<td>10 h 2 h 10 h 2 h</td>
</tr>
</tbody>
</table>
Olate oxidation was measured as $^{14}$CO$_2$ production from [1-$^{14}$C]oleate, using the same experimental design as for measurement of glucose oxidation.

**Immunoblotting.** After culture, 30 islets were lysed in lysate buffer (1% Triton, 0.1% SDS). Samples were analyzed using 12% sodium dodecyl sulfate-polycrylamide gel electrophoresis (SDS-PAGE) and run for 1 h at 150 V and then transferred to nitrocellulose for 1 h at 250 mA. The membrane was blocked for 2 h at room temperature with 5% fat-free milk and 0.1% Tween-20 in Tris-buffered saline. The membrane was incubated with SNAP-25 mouse monoclonal antibody (diluted 1:5,000) for 1 h at room temperature, followed by incubation with secondary antibody (diluted 1:2,000) for 1 h at room temperature, followed by incubation with secondary antibody (diluted 1:5,000) for 1 h at room temperature. Immunoreactive bands were visualized by ECL (Amersham International).

**Statistical analysis.** Results are expressed as means ± SE of the number of experiments indicated in the figure legends. Concentration-response curves were analyzed using computerized curve-fitting software (Graphpad Prism, Windows software). The data of RT-PCR experiments are shown as the average of three cycles (e.g., 30, 32, 34 cycles) between each gene and experiment. Significance testing was carried out by Student’s paired t-test or, in case of multiple comparisons, by ANOVA. A $P$ value of <0.05 was considered significant.

**RESULTS**

Not only continuous but also intermittent exposure to diazoxide increases insulin release. Coculture with diazoxide at 27 mmol/l glucose for 48 h (protocol HD48/48; Fig. 1) markedly depressed insulin accumulation into the RPMI medium (by 67 ± 4%, $n = 4$, $P < 0.01$). The continuous coculture with diazoxide markedly enhanced postculture insulin release in response to 27 mmol/l glucose and moderately at basal levels (3.3 mM) of glucose (Fig. 2A). Previous diazoxide also increased islet insulin contents by 52% (Fig. 2B).

A 2-h exposure to diazoxide every 12 h (the glucose concentration being continuously kept at 27 mM, protocol HD8/48; Fig. 1) did not markedly decrease insulin accumulation into the RPMI medium [by 11.3 ± 4.6%, $n = 4$, not significant (NS) vs. culture without diazoxide]. However, the intermittent exposure to diazoxide significantly enhanced the response to 27 mmol/l glucose (Fig. 2A). Islet insulin contents were not affected (Fig. 2B).

When the last 2-h period of exposure to diazoxide was omitted (protocol HD6/48; Fig. 1), there was no significant effect on postculture insulin secretion (Fig. 2A). Neither was there any effect of a final, solitary 2-h exposure to diazoxide (protocol HD2/48; Fig. 1).

The effects of intermittent diazoxide were compared with effects of lowering the glucose concentration during corresponding time intervals. A 2-h exposure to 5.5 mmol/l glucose every 12 h (protocol HL8/48; Fig. 1) did not affect the postculture insulin response to glucose (Fig. 2).

**Aftereffects of intermittent and continuous diazoxide differ qualitatively.** Both intermittent and continuous exposure to diazoxide caused an increase in the calculated maximal response to glucose vs. culture at high glucose only (Fig. 3). Both modalities also increased postculture responses to tolbutamide and to KCl (Table 2). However, only continuous diazoxide.

**Table 1. Sequence of oligonucleotide primers for PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Size, bp</th>
<th>5'-Oligonucleotide</th>
<th>3'-Oligonucleotide</th>
<th>GenBank No.</th>
<th>Annealing Temperature, °C</th>
<th>Cycle</th>
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<td>UCP2</td>
<td>345</td>
<td>ATT GCA GGA GAG GAA GGG</td>
<td>CAA GGG GAG GAA GGA AGG</td>
<td>AB00613</td>
<td>55</td>
<td>30–34</td>
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<tr>
<td>Kir6.2</td>
<td>199</td>
<td>CAT GGA GAA CAG TCT GCT GGG</td>
<td>CAG GTA GAA GGT GCG GCC</td>
<td>U44897</td>
<td>59</td>
<td>38–42</td>
</tr>
<tr>
<td>SUR1</td>
<td>178</td>
<td>TGA AGC AAC TGC CTC CAT C</td>
<td>GAA GCT TTT CCG TGT TGT C</td>
<td>L40624</td>
<td>59</td>
<td>38–42</td>
</tr>
<tr>
<td>VDCCα1</td>
<td>155</td>
<td>TTG TCA GGG AAT GGC GGC</td>
<td>AGC ACA CTT GTC CAG CCC</td>
<td>D38101</td>
<td>59</td>
<td>38–42</td>
</tr>
<tr>
<td>Kv2.1</td>
<td>430</td>
<td>GAA TCT GTG GAC ACT AAG TAG C</td>
<td>GGA GAA AGA ATT GAG TCT TTT T</td>
<td>X16476</td>
<td>58</td>
<td>38–42</td>
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<tr>
<td>β-Actin</td>
<td>349</td>
<td>GGT AAA GAC CTA TAT GGC AA</td>
<td>AGG CAT GGG AAA TGT GTC AT</td>
<td>J00691</td>
<td>55</td>
<td>30–34</td>
</tr>
</tbody>
</table>

Kv2.1, voltage-dependent K$^+$ 2.1 channel; SUR1, sulfonylurea receptor 1; VDCCα1, voltage-dependent Ca$^{2+}$ channel-α1; UCP2, uncoupling protein-2.

Fig. 2. A: postculture insulin release in the presence of 3.3 mmol/l (open bars) or 27 mmol/l glucose (filled bars) for 60 min. Islets were first cultured under various conditions (see Fig. 1). B: insulin content in islets (gray bars). Results are expressed as means ± SE of 4–13 experiments (A) or 4 experiments (B). *$P < 0.05$. 

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exposure enhanced the insulin response to the phosphodiesterase inhibitor IBMX (Table 2).

We tested for effects of previous diazoxide on the K<sub>ATP</sub>-independent effect of glucose. As in previous studies (16), this was done by assessing the response to a high glucose concentration (27 mmol/l) in the presence of KCL and diazoxide, i.e., during conditions that bypass a regulatory influence on K<sub>ATP</sub> channels. During these conditions, the continuous diazoxide exposure led to improvement of a K<sub>ATP</sub>-independent effect of glucose (Fig. 4). In contrast, intermittent diazoxide exposure failed to exert such an effect.

**KCl abrogates the secretory effect of intermittent diazoxide.**

Addition of 20 mmol/l KCl for the last 2 h of culture increased the accumulation of insulin into culture medium in both the absence and the presence of the protocol of intermittent diazoxide (Fig. 5A). The 2-h exposure to KCl did not significantly affect the postculture insulin response of islets cultured without diazoxide. However, KCl abrogated the postculture insulin response to 27 mmol/l glucose from islets intermittently exposed to diazoxide. Addition of KCl to islets cultured without diazoxide failed to affect the postculture insulin response (Fig. 5B). However, cycloheximide abrogated the enhancing effect of intermittent diazoxide on the postculture insulin response (Fig. 5B). Addition of cycloheximide did not affect insulin contents (4.0 ± 0.3 mU/islet, protocol HD8/48; 3.8 ± 0.5 mU/islet, protocol HD8/48 + cycloheximide; n = 10, means ± SE; Fig. 1). Cycloheximide did not abrogate the enhancing effect of continuous diazoxide (Fig. 5B).

**Continuous and intermittent exposure to diazoxide increases islet ATP/ADP ratios.** Coculture with diazoxide did not significantly affect ATP contents of islets (Fig. 6A). However, both continuous and intermittent diazoxide treatments increased ATP/ADP ratios (Fig. 6B).

**Previous diazoxide does not affect Kir6.1, SUR1, VDCCα1, and Kv2.1 mRNA expression.** We examined gene expression for genes that could potentially be involved in the K<sub>ATP</sub>-dependent pathway in β-cells. None of the genes tested (Kir6.2, SUR1, VDCCα1, and Kv2.1 mRNA) were influenced by either continuous or intermittent diazoxide (Table 3).

**Continuous but not intermittent diazoxide reduces UCP2 mRNA.** Increased expression of UCP2 is associated with decreased glucose-induced insulin secretion and vice versa (15). It was therefore of interest to test for effects of diazoxide on UCP2 mRNA. We found that continuous but not intermittent diazoxide reduces UCP2 mRNA (Table 3).

**Previous diazoxide does not affect glucose oxidation but decreases oleate oxidation.** Neither continuous nor intermittent exposure of islets to diazoxide affected postculture oxidation of glucose (Table 4). However, both conditions inhibited oleate oxidation (Table 4). This effect was not reproduced by exposing islets to diazoxide only during the final 2-h period of culture.

**Intermittent diazoxide affects exocytotic SNARE protein SNAP-25.** The possible involvement of exocytotic proteins in the secretory effects of intermittent diazoxide was probed by

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**Fig. 3.** Insulin release in the presence of 1.0, 3.3, 5.5, 11.8, 16.8, and 27 mmol/l glucose for 60 min in islets cultured with 27 mmol/l glucose for 48 h (■, HG), 27 mmol/l glucose and 325 μmol/l diazoxide for 48 h (▲, HD48/48), and 27 mmol/l glucose and 2-h incubation of 325 μmol/l diazoxide every 12 h for 48 h (●, HD4/48). Results are expressed as means ± SE of 4 experiments. *P < 0.05 vs. HG.

**Fig. 4.** Insulin response to 27 mmol/l glucose in the presence of 20 mmol/l KCl and 325 μmol/l diazoxide for 60 min. Results are expressed as means ± SE of 4 experiments. *P < 0.05.

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**Table 2. Effects of KCl, tolbutamide, and IBMX on insulin secretion**

<table>
<thead>
<tr>
<th>Protocols of Culture Condition</th>
<th>Insulin Release in Final Incubation. μU/islet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KCl (20 mmol/l)</td>
</tr>
<tr>
<td>HG</td>
<td>89 ± 7</td>
</tr>
<tr>
<td>HD8/48</td>
<td>128 ± 17*</td>
</tr>
<tr>
<td>HD48/48</td>
<td>153 ± 7*</td>
</tr>
</tbody>
</table>

Values are means ± SE from 4–6 experiments. For protocol abbreviations see Fig. 1. *P < 0.05 vs. HG.
measuring SNAP-25, which is a key SNARE protein (4). Relative to culture at glucose only, SNAP-25 was decreased by intermittent diazoxide, and this effect was antagonized by KCl (Fig. 7).

Previous diazoxide does not affect apoptosis. The intermittent and continuous exposure of islets to diazoxide did not influence apoptosis [no addition: 0.193 ± 0.008 optical density (OD), intermittent diazoxide: 0.181 ± 0.004 OD, continuous diazoxide: 0.204 ± 0.036 OD, n = 4–6, means ± SE].

DISCUSSION

This study has tested and documented, to our knowledge for the first time, beneficial effects of intermittent β-cell rest as achieved by exposure to diazoxide in vitro. Such effects are important in a clinical perspective. Being operational, they could influence therapy in type 2 diabetes, for instance the choice between sulfonylurea of the short-acting and long-acting type. Conceptually, these results introduce a new dimension of the impact of long-term elevated glucose on β-cell function.

We compared the beneficial effects of intermittent diazoxide with those of a 48-h continuous exposure to the drug. We find both similarities and qualitative differences. Similarities included the insulin dose-response curve to postculture stimulation by glucose, the enhancement of tolbutamide and KCl-induced insulin secretion, the absence of effect on glucose oxidation, and a reduction of fatty acid oxidation (discussed further below). Important differences were seen with regard to

<table>
<thead>
<tr>
<th>mRNA Gene/β-Actin</th>
<th>HG</th>
<th>HD8/48</th>
<th>HD8/48</th>
</tr>
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<tbody>
<tr>
<td>Kv2.1</td>
<td>0.96±0.03</td>
<td>1.01±0.06</td>
<td>0.93±0.19</td>
</tr>
<tr>
<td>Kir6.2</td>
<td>0.97±0.17</td>
<td>1.03±0.12</td>
<td>1.06±0.89</td>
</tr>
<tr>
<td>SUR1</td>
<td>1.22±0.60</td>
<td>1.09±0.17</td>
<td>1.16±0.19</td>
</tr>
<tr>
<td>VDCCα1</td>
<td>0.80±0.04</td>
<td>0.76±0.03</td>
<td>0.81±0.12</td>
</tr>
<tr>
<td>UCP2</td>
<td>1.16±0.05</td>
<td>0.99±0.05</td>
<td>1.15±0.06</td>
</tr>
</tbody>
</table>

Values are means ± SE from 3–17 experiments. * P < 0.05 vs. HG.
islet insulin contents (increased after continuous, unaltered after intermittent, diazoxide). Furthermore, $K_{\text{ATP}}$-independent effects by glucose on insulin secretion were enhanced by continuous diazoxide exposure in postculture incubations but were unaltered by intermittent diazoxide exposure.

A further difference was observed in relation to IBMX-induced insulin secretion, which was enhanced by continuous, but not by intermittent, diazoxide exposure. cAMP is known to induce insulin secretion, which was enhanced by continuous, diazoxide exposure in postculture incubations but testing but not by intermittent diazoxide exposure.

Kv2.1 mRNA was, however, negative. The enhancing effect of previous diazoxide, which becomes operative on renewed blocking of insulin secretion. Such a priming effect could imply accelerated biosynthesis of proteins important in stimulus secretion coupling. Such a mechanism is compatible with the observation that inhibition of protein biosynthesis during the last 2 h of culture abrogated the beneficial effect of intermittent diazoxide. However, the degradative process of important proteins could also be subject to priming. Such a mechanism could play a part in the intriguing finding that intermittent diazoxide reduces the concentration of the exocytotic protein SNAP-25 and that KCl reverses this effect.

A possible link to effects on the $K_{\text{ATP}}$-dependent modulation of insulin secretion is provided by SNAP-25 and other SNARE proteins being intimately associated with Ca$^{2+}$ channel complexes, giving rise to the term “exosomes” (1). SNAP-25 is also intimately associated with Kv2.1 channels in islets (7).

Further studies are needed to clarify these issues.

Replacing intermittent diazoxide exposure with corresponding periods of low (5.5 mmol/l) glucose did not reproduce the beneficial effects of diazoxide. At this concentration of glucose, $K_{\text{ATP}}$-dependent pathway secretion may not be completely absent. This could account for the fact that blocking insulin secretion by diazoxide occurs with shorter latency and more effectively than acute lowering of the ambient glucose concentration to a physiological one (2). It is possible that a difference in insulin blocking effectiveness explains the failure of intermittent low glucose to mimic the effects of intermittent diazoxide. Of possible relevance for the present results is also that 5.5 mmol/l glucose is, in rat islets, known to be a suboptimal concentration for upholding nutritional demands during culture.

In conclusion, we have demonstrated that intermittent diazoxide exposure has beneficial effects on $K_{\text{ATP}}$-dependent glucose-induced insulin secretion. Our studies in type 2 diabetes patients support the notion that intermittent $\beta$-cell rest is beneficial. The inclusion of KCl during the last 2 h of diazoxide obliterated the permissive effect of the last 2-h period of diazoxide. The permissive effect of the last 2 h of diazoxide, therefore, did not seem linked to a drug effect per se but instead to the blocking effect on depolarization and/or the insulin-secretory process.

Our results are complex as to the functional importance of the last 2 h of diazoxide in relation to the previous periods of diazoxide. A single 2-h period of diazoxide exposure at the end of the 48-h culture period with elevated glucose was without effects on subsequent glucose-induced insulin secretion; yet the last 2-h period of diazoxide was required to demonstrate beneficial effects of previous intermittent exposure to diazoxide.

![Fig. 7. S-nitroso-N-acetyl-D,L-penicillamine (SNAP)-25 protein content in islets. Results are expressed as means ± SE of 8–13 experiments. *$P < 0.05$.](http://ajpendo.physiology.org/ by 10.220.33.5 on September 8, 2017 http://ajpendo.physiology.org/ Downloaded from)
favorable for β-cell function (14). This conclusion based on the clinical observations is compatible with data from the present in vitro study.

GRANTS

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REFERENCES