Reduction of reactive oxygen species ameliorates metabolism-secretion coupling in islets of diabetic GK rats by suppressing lactate overproduction

Short running title: ROS and lactate production in GK rat

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ABSTRACT

We previously demonstrated that impaired glucose-induced insulin secretion (IS) and ATP elevation in islets of Goto-Kakizaki (GK) rats, a non-obese model of diabetes, were significantly restored by 30–60 min suppression of endogenous reactive oxygen species (ROS) overproduction. In this study, we investigated the effect of longer (12 h) suppression of ROS on metabolism-secretion coupling in β-cells by exposure to tempol, a superoxide dismutase mimic, plus ebselen, a glutathione peroxidase mimic (TE-treatment). In GK islets, both H$_2$O$_2$ and superoxide were sufficiently reduced and glucose-induced IS and ATP elevation were improved by TE-treatment. Glucose oxidation, an indicator of Krebs cycle velocity, also was improved by TE-treatment at high glucose, whereas glucokinase activity, which determines glycolytic velocity, was not affected. Lactate production was markedly increased in GK islets and TE-treatment reduced lactate production and protein expression of lactate dehydrogenase and hypoxia-inducible factor 1α (HIF1α). These results indicate that the Warburg-like effect, which is characteristic of aerobic metabolism in cancer cells by which lactate is overproduced with reduced linking to mitochondria metabolism, plays an important role in impaired metabolism-secretion coupling in diabetic β-cells and suggest that ROS reduction can improve mitochondrial metabolism by suppressing lactate overproduction through inhibition of HIF1α stabilization.
In pancreatic β-cells, intracellular glucose metabolism regulates exocytosis of insulin granules according to metabolism-secretion coupling in which ATP production in mitochondria plays an essential role (1). Reduction of mitochondrial ATP production causes impairment of glucose-induced insulin secretion in various conditions (2).

Reactive oxygen species (ROS) such as superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) are normal byproducts of glucose metabolism including glycolysis and mitochondrial oxidative phosphorylation (3). In pancreatic β-cells, ROS production via non-mitochondrial and mitochondrial pathways has been proposed. In the mitochondrial pathway, ROS is generated in the electron transport chain associated with the mitochondrial membrane potential (4). However, in pathophysiological conditions, NADPH oxidase, an important nonmitochondrial ROS source, may play an important role in ROS generation in β-cells (5). Antioxidant capacity in β-cells is very low because of weak expression of antioxidant enzymes such as catalase (CAT), glutathione peroxidase (Gpx), and superoxide dismutase (SOD) in pancreatic islets compared with that in various other tissues (6, 7), which suggests vulnerability of β-cells to ROS. Gene expression profiling in islets revealed that SOD, which metabolizes O$_2^-$ to H$_2$O$_2$, was 30-40% and Gpx, which metabolizes H$_2$O$_2$ to H$_2$O, was 15%, of that in liver. Moreover, CAT was not detectable in islets (7).

In β-cells, ROS is one of the most important factors that impair metabolism-secretion coupling (1). Exposure to exogenous H$_2$O$_2$, the most abundant ROS, reduces glucose-induced insulin secretion by impairing mitochondrial metabolism in β-cells (8). We have proposed that endogenous overproduction of ROS involving activation of Src, a non-receptor tyrosine kinase, plays a significant role in impaired metabolism-secretion coupling in islets of diabetic Goto-Kakizaki (GK) rats (9-11). Suppression of overproduction of ROS for 30–60 min by exposure to ROS scavengers
and by suppression of Src activity restores impaired glucose-induced insulin secretion and ATP elevation in GK rat islets (9, 10). However, the effect of reduction of overproduction of ROS for a longer duration on impaired metabolism-secretion coupling in diabetic β-cells remains unknown.

In the present study, we investigated the effects of 12 h suppression of endogenous ROS production on impaired metabolism-secretion coupling in β-cells by exposing cell-permeable antioxidant enzyme mimics including tempol, a SOD mimic (12), and ebselen, a GPx mimic (13), which are commonly used in the field of diabetology without cytotoxic effects (14,15). Our results indicate that 12 h suppression of ROS improves metabolism-secretion coupling by mechanism different from that involved in improvement by ROS reduction for 30~60 min.
RESEARCH DESIGN AND METHODS

Materials.
Ebselen was purchased from Calbiochem (La Jolla, CA). HEPES, KCl, EGTA, glucose, NaCl, NaHCO₃, HClO₄, Na₂CO₃, H₂O₂, BSA, and the substrates used in ATP production except glycerol phosphate were purchased from Nacalai (Kyoto, Japan). [U⁻¹⁴C]-glucose was obtained from GE Healthcare (Uppsala, Sweden). Lactate dehydrogenase (EC 1.1.1.27) and Dowex 1x8 anion exchange resin (formate) (50-100 mesh) were obtained from Wako (Osaka, Japan). HIF1α inhibitor [3-(2-(4-Adamantan-1-yl-phenoxy)-acetylamino)-4-hydroxybenzoic acid methyl ester] was obtained from Merck Millipor (Darmstadt, Germany). All other reagents were obtained from Sigma Chemicals (St. Louis, MO).

Animals.
Male Wistar and Goto-Kakizaki (GK) rats were obtained from Shimizu (Kyoto, Japan). All experiments were carried out with rats aged 7-10 weeks. Body weight of GK rats used in the experiments was similar to that of Wistar rats [231 ± 3 (means ± SE), Wistar vs. 217 ± 3 g, GK, not significant]. Non-fasting plasma glucose of GK rats was higher compared to that of Wistar rats [5.72 ± 0.12 (means ± SE), Wistar vs. 11.57 ± 0.58 mmol/l, GK, P<0.01]. The animals were maintained and used in accordance with the guidelines of the animal care committee of Kyoto University.

Islet isolation and culture.
Pancreatic islets were isolated from Wistar and GK rats by collagenase digestion as described previously (16). Isolated islets were washed with Krebs-Ringer bicarbonate buffer (KRBB) containing 2.8 mmol/l glucose, and cultured for 12 hours in RPMI-1640 medium containing 10% fetal calf serum (FCS) and 5.5 mmol/l glucose without or with
10 mmol/l tempol (T), 10 µmol/l ebselen (E), or combination of T and E (TE-treatment) at 37°C in humidified air containing 5% CO₂. Insulin content of GK islets was similar to that of Wistar islets [20.0 ± 2.2 (means ± SE), Wistar vs. 23.0 ± 2.6 ng/islet, GK, not significant].

**Measurement of superoxide and H₂O₂ generations.**

Superoxide (O₂⁻) and the H₂O₂ production were measured at the end of the 12 h culture in conditions described above. O₂⁻ generation was detected by nitroblue tetrazolium (NBT) assay (17) using KRBB supplemented with 0.2 % BSA (fraction V) and 10 mmol/l HEPES adjusted to pH 7.4 (KRBB medium). Groups of 100 islets were batch incubated in tubes containing 0.5 ml KRBB medium supplemented with 5.5 mmol/l glucose with 0.2% (wt/vol) NBT at 37°C for 30 min. After the islets were centrifuged (1,000 rpm for 2 min at 4°C), the supernatant was removed, and formazan-NBT (NBT reduced) was dissolved in 100 µl 50% (vol/vol) acetic acid by sonication (5s pulse, five times). The sonicate was briefly centrifuged, and absorbance of the supernatant was measured at 560 nm using a spectrofluorometer (Shimadzu RF-5000, Kyoto, Japan).

H₂O₂ release from islets was measured according to the method previously described (18). Briefly, islets were cultured for 12 h in various conditions as described above. Groups of 150 islets were batch incubated for 60 min in KRBB medium containing 5.5 mmol/l glucose with 0.1 mg/ml horseradish peroxidase (type II) and 0.44 mmol/l homovanillic acid. After the islets were centrifuged (1,000 rpm for 2 min at 4°C), the supernatant was removed. Fluorescence of the supernatant was measured at excitation and emission of 315 and 425 nm, respectively, using a spectrofluorometer (Shimadzu RF-5000). Standard curves were produced using samples containing known amounts of H₂O₂ without islets.

**Measurement of insulin release, ATP contents and glucose oxidation.**
Insulin release from islets was monitored using batch incubation as described previously (16). ATP content in islets was determined as previously described (9). ATP contents were measured using ENLITEN luciferin-luciferase solution (Promega, Madison, WI) by luminometer (GloMax 20/20n; Promega). Glucose oxidation was carried out using the previously described method (22).

**Intraperitoneal glucose tolerance tests (ip-GTTs).**

After GK rats were divided into two groups, one group received intraperitoneal injection of tempol (50 mg/kg) and ebselen (10 mg/kg) twice (12 h and 6 h before ip-GTTs) during a 15 h fast (TE); the other group was treated similarly with vehicle alone (C). After these treatments, ip-GTTs (1 g/kg body wt) were performed. Plasma glucose and plasma insulin levels were measured in samples taken at the indicated times. Plasma glucose levels were determined by the glucose oxidase method. Plasma insulin levels were determined using enzyme immunoassay (Shibayagi, Gunma, Japan).

**Immunoblot analysis.**

Preparations for whole-islet lysate and for mitochondrial fraction were described previously (19, 20). Western blotting was performed as previously described (19) with the following antibodies: mouse monoclonal anti-complex I (39 kDa subunit), anti-complex III (core II), anti-complex IV (subunit I), and anti-complex V (subunit α) of mitochondrial respiratory chain (1:1000) from Invitrogen (Carlsbad, CA), mouse anti-β-actin (1:5000) from Sigma, rabbit polyclonal anti-LDH-A (1:1000) from Cell Signaling (Danvers, MA), rabbit anti-PDK1 (1:1000) and rabbit anti-HIF1α (1:250) from Abcam (Paris, France), rabbit anti-UCP2 (1:200) from Chemicom (Temecula, CA), mouse anti-hsp-60 (1:5000) from BD Biosciences (Franklin Lakes, NJ), rabbit anti-PK-M2 (1:2500) from Novus biological, LLC (Littleton, CO), and horseradish peroxidase-conjugated anti-rabbit and mouse antibody (1:5000) from GE Healthcare.
Proteins were detected using an enhanced chemiluminescence (ECL) system (GE Healthcare). Protein density was quantified by densitometric analysis using Multi Gauge software (Fujifilm, Tokyo, Japan).

**Measurements of enzyme activities.**

After islets were cultured with or without TE for 12 h, whole islet homogenates and mitochondrial fractions from cultured islets were obtained. Glucokinase and hexokinase activities were determined using whole islet homogenates by NADH formation in an enzyme reaction as previously described (21). Activity of mitochondrial glycerol phosphate dehydrogenase (mGPDH) in whole islet homogenates of Wistar and GK rats was measured by reduction of 2-p-iodo-3-p-nitro-5-phenyltetrazolium to indoformazan as previously described (23). Activity of mGPDH in mitochondrial fraction from GK islets was measured by the method based on the generation of $^3$HOH from L-[2-$^3$H] glycerol 3-phosphate (24). Briefly, after mitochondrial fractions were incubated at 37ºC for 30 min with 2.5 mCi/mmol L-[2-$^3$H] glycerol 3-phosphate and the reaction was stopped by the addition of antimycin A, the mixture was immediately applied to a column of Dowex 1-X8 (formate) which was washed with 0.5 ml of water. The radioactivity of the column effluent was measured by liquid scintillation counter.

**Measurement of mitochondrial ATP production.**

Mitochondrial fraction from islets and measurement of ATP production were performed as previously described (20).

**Measurement of lactate production.**

Lactate production was measured as previously described (25). Briefly, after preincubation, groups of 20 islets were batch incubated in KRBB medium containing 2.8 and 16.7 mmol/l glucose for 60 min at 37ºC. 200 µl of the supernatant was mixed with 0.5 ml of 0.5 mol/l glycine / 0.4 mol/l hydrazine buffer (pH 9.0) containing 2.4
mmol/l NAD and 7.2 units/ml lactate dehydrogenase, and was incubated for 30 min at 37°C. Fluorescence of NADH was then measured at excitation of 340 nm and emission of 450 nm. Standard curves were produced from samples containing known amounts of lactate.

**Statistical analysis.**

The data are expressed as means ± SE. Statistical significance was determined by unpaired Student’s *t*-test. *P* < 0.05 was considered significant.
RESULTS

O$_2^-$ and H$_2$O$_2$ production during culture with antioxidant mimics.

Tempol, a SOD mimic, decreased O$_2^-$ production in Wistar (Fig. 1A) and GK islets (Fig. 1B), but had no effect on H$_2$O$_2$ production in either of the islets (Fig. 1CD). Ebselen, a GPx mimic, had no reducing effect on O$_2^-$ and H$_2$O$_2$ production in either of the islets (Fig. 1). Co-treatment with tempol and ebselen (TE-treatment) reduced O$_2^-$ production by 27.0% but had no effect on H$_2$O$_2$ production in Wistar islets (Fig. 1AC). On the other hand, in GK islets, TE-treatment prominently reduced O$_2^-$ and H$_2$O$_2$ production by 65.0% and 84.6%, respectively (Fig. 1BD). Taken together, these findings indicate that TE-treatment is effective in reducing oxidative stress in GK islets.

Effect of TE-treatment on insulin secretion.

The effect of antioxidant mimics on β-cell function was investigated. In the presence of 16.7 mmol/l glucose, insulin secretion (IS) from GK islets was reduced compared with that from control Wistar rats (1.01 ± 0.06, GK vs. 2.32 ± 0.11 ng/30min/islet, Wistar, P<0.01). While TE-treatment had no effect on IS from Wistar islets (Fig. 2A), it restored high glucose-induced IS (Fig. 2B) and ATP content (Fig. 2C) in GK islets. We demonstrated previously that impaired glucose-induced IS in GK islets was significantly restored by acute exposure to PP2, a Src inhibitor (9). To examine the involvement of Src inhibition in improvement of glucose-induced IS after TE-treatment, TE-treated GK islets were incubated for 30 min with or without 10 µmol/l PP2 in the presence of 16.7 mmol/l glucose. In TE-treated GK islets, 30 min exposure to PP2 prominently enhanced IS at high glucose (1.71 ± 0.07, control vs. 4.06 ± 0.22 ng/30min/islet, PP2, P<0.01), which implies involvement of an independent mechanism of Src inhibition in the improvement of glucose-induced IS by chronic TE-treatment.
To investigate effects of TE-treatment on β-cell function in vivo, glucose tolerance test was assessed after intraperitoneal TE administration (Table 1). In GK rats, TE treatment decreased the glucose level at 15 min and 30 min, and increased insulin levels at 30 min after glucose loading. Studies using isolated islets from GK rats after TE-treatment in vivo revealed that high glucose-induced insulin secretion was increased by TE-treatment (Table 2).

**Effect of TE-treatment on expression of UCP2 and mitochondrial respiratory chain proteins.**

Immunoblot analysis showed that TE-treatment had no effect on protein levels of UCP2 in protein extracts of islet mitochondrial fraction (supplementary Fig. 1A) and on those of complex I, III, IV, and V of the mitochondrial respiratory chain in lysates of whole islets (supplementary Fig. 1B) in both GK and Wistar islets.

**Effect of TE-treatment on glucose metabolism.**

TE-treatment had no effect on glucokinase and hexokinase activities, which determines velocity of glycolysis in β-cells (26), in GK and Wistar islets (Table 3). By TE-treatment, glucose oxidation at high glucose, an indicator of Krebs cycle velocity, was not affected in Wistar islets, while it was significantly increased in GK islets (25.4 ± 4.1, control vs. 55.8 ± 12.2 pmol/islet/90 min, TE, P<0.05) (Fig. 3).

**Effect of TE-treatment on ATP production and on activity of mitochondrial glycerol phosphate dehydrogenase.**

ATP production by mitochondria from control and TE-treated islets of Wistar and GK rats was measured in the presence of various substrates and inhibitors (Table 4)
including succinate (electron transfer at complex I by NADH generation in the Krebs cycle and at complex II directly), succinate with rotenone (electron transfer at complex II and inhibition of electron transfer through NADH at complex I), glutamate plus malate (electron transfer mainly at complex I by NADH generation derived from reaction via glutamate dehydrogenase), pyruvate plus malate (electron transfer mainly at complex I by NADH generation derived from reaction via pyruvate dehydrogenase), ascorbate plus TMPD (electron transfer at complex IV directly), and glycerol-3-phosphate [electron transfer at complex II by FADH$_2$ generation derived from reaction via mitochondrial glycerol phosphate dehydrogenase (mGPDH)]. Control and TE-treated mitochondria showed similar rates of ATP production for all substrates tested, except for glycerol-3-phosphate. TE-treatment promotes the rate of mitochondrial ATP production in the presence of glycerol-3-phosphate in Wistar and GK islets 3.3 and 2.2 fold, respectively. In measurement of activity of mGPDH using whole islets homogenates, the value in GK islets was lower compared with that of Wistar islets, and enhancement of the activity by TE-treatment was not significant in either Wistar or GK islets (Table 3). However, in measurement of activity of mGPDH using intact mitochondria, TE-treatment increased mGPDH activity in GK mitochondria (Table 3).

**Lactate production and protein expression of lactate dehydrogenase and HIF1α.**

Lactate production in GK islets was significantly higher compared to that in Wistar islets at both basal and stimulated levels of glucose (at 2.8 mmol/l glucose: 1.32 ± 0.15, Wistar vs. 8.15 ± 1.36 GK, $P<0.01$; at 16.7 mmol/l glucose: 3.09 ± 0.33, Wistar vs. 14.08 ± 1.68, GK, $P<0.01$) (Fig. 4A). TE-treatment suppressed lactate production at both basal and stimulated levels of glucose in both Wistar and GK islets; the most
prominent reduction in these conditions was at 16.7 mmol/l glucose in GK islets (14.08 ± 1.68, control vs. 5.71 ± 0.79, TE, P<0.01). Protein expression levels of HIF1α, a potential upstream regulator of lactate dehydrogenase (LDH), in INS-1 cells were gradually increased over 12 h in the presence of 200 μmol/l CoCl2, a chemical inducer of HIF1α (27) (supplementary Fig. 2). TE-treatment time-dependently suppressed HIF1α levels in GK islets, and the reduction was significant after 9 h TE-treatment (~30% reduction at 9 h; ~50% reduction at 12 h) (Fig. 4B). Protein expression levels of HIF1α and HIF1α downstream targets including LDH-A and PDK-1 were examined 12 h after TE-treatment. In GK islets, TE-treatment significantly decreased HIF1α, LDH-A, and M2-PK expression (HIF1α: ~50% reduction; LDH-A: ~30% reduction; M2-PK: ~30% reduction), but did not affect PDK1 expression (Fig. 4C and supplementary Fig. 3).

**Effect of HIF1α inhibitor on insulin secretion and lactate production in GK islets**

Insulin secretion in the presence of 16.7 mmol/l glucose from GK islets was enhanced by HIF1α inhibitor-treatment (1.34 ± 0.07, control vs. 1.77 ± 0.09 ng/30min/islet, HIF1α inhibitor, P<0.01). Lactate production in the presence of 2.8 mmol/l glucose and 16.7 mmol/l glucose was reduced by HIF1α inhibitor-treatment (2.8 mmol/l glucose: 12.44 ± 1.12, control vs. 9.46 ± 0.89 pmol/islet, HIF1α inhibitor, P<0.01; 16.7 mmol/l glucose: 15.39 ± 1.02, control vs. 12.42 ± 1.40 pmol/islet, HIF1α inhibitor, P<0.01).
DISCUSSION

The present study demonstrates that combination treatment by tempol, a SOD mimic and ebselen, a GPx mimic (TE-treatment), efficiently suppressed both H$_2$O$_2$ and O$_2^-$ production in GK islets. Moreover, impaired glucose-induced insulin secretion and ATP elevation in GK islets were significantly improved by TE-treatment but were not improved by tempol or ebselen alone. These results are compatible with previous studies in which co-expression of the antioxidant enzymes in a β-cell line was more efficient than expression of either single enzyme in reducing of ROS production and oxidative injury (7, 28, 29).

We then examined the precise mechanisms of the improvement in glucose-induced ATP elevation in GK islets by TE-treatment. UCP2 decreases mitochondrial ATP production by reducing mitochondrial hyperpolarization derived from an increase in mitochondrial proton conductance. UCP2 negatively regulates metabolism-secretion coupling in β-cells (30,31). However, regulation of UCP2 expression may not play an important role in the mechanism, as the UCP2 expression level was not affected in GK islets by TE-treatment. Western blot analysis revealed that the expressions of respiratory chain proteins including complex I, III, IV, and V were not affected by TE-treatment. In addition, the relative mRNA levels of nuclear factors including NRF-1, NRF-2 and TFAM, which affect mitochondrial biogenesis, also were not altered by TE-treatment (Data not shown). These results indicate that regulation of mitochondrial mass is not involved in the mechanism.

ATP production is driven by the H$^+$ gradient across the mitochondrial membrane generated by transport of high-energy electrons in the respiratory chain. These electrons are derived from NADH and FADH$_2$ derived from the Krebs cycle in the matrix and/or transferred from the cytosol by the shuttle system. To determine which
step in mitochondrial carbohydrate metabolism is affected in TE-treated islets, ATP production in the presence of various substrates and inhibitors was measured using mitochondrial fractions from the cultured islets. In the presence of glycerol-3-phosphate (G3P), ATP production was increased by TE-treatment in both Wistar and GK mitochondrial fraction. Transfer of a reduced equivalent from cytosol to mitochondria by the glycerol phosphate (GP) shuttle may well play a more important role in mitochondrial ATP production in islets than in other tissues, since activity of mGPDH, a key enzyme in the GP shuttle is ~60-fold higher in islets than in other tissues (24). In addition, decreased activity is observed in diabetic islets (32). While an increase in activity of mGPDH by TE-treatment was detected, the increase was similar in mitochondrial fraction from both GK and Wistar islets. Therefore, improvement of mGPDH activity alone may not account for the improvement in glucose-induced ATP elevation in GK islets by TE-treatment.

TE-treatment did not affect activity of glucokinase, a rate-limiting enzyme in glycolysis (26). In contrast, glucose oxidation at high glucose, which reflects Krebs cycle velocity, was improved in GK islets by TE-treatment. These results suggest that coupling between glycolysis and mitochondrial oxidation, which plays an important role in glucose-induced insulin secretion (33), is improved by TE-treatment. Lactate production weakens coupling between glycolysis and mitochondrial oxidation by decreasing the pyruvate supply to mitochondria. In several studies, overexpression of LDH-A in β-cells attenuated glucose-induced insulin secretion (34-36) with an increase in lactate production (34,37). In addition, lactate release from β-cells increases in parallel with a decrease in the glucose oxidation rate (36). These findings support the hypothesis that regulation of lactate production may play an important role in improvement of metabolism-secretion coupling by TE-treatment. In the present study,
lactate production in GK islets was higher compared to that in control, as shown in a previous study (38), and it was reduced by TE-treatment. The protein expression level of LDH-A, an enzyme that catalyzes reaction from pyruvate and NADH to lactate and NAD$^+$, was 8.6-fold higher in GK islets compared to control Wistar islets, and was reduced by ~30% after TE-treatment. In non-diabetic pancreatic β-cells, low LDH activity together with high mGPDH activity may act as a device to transfer NADH in the cytosol to the mitochondria and reduce lactate production by decreasing the NADH level in cytosol (39). In GK islets, enhanced LDH activity and reduced mGPDH activity might produce lactate overproduction and reduce mitochondrial oxidation by decreasing the supply of pyruvate and reducing equivalents to mitochondria and by an increase in the availability of cytosolic NADH to produce lactate from pyruvate as well. In this case, reduced LDH activity and increased mGPDH activity by TE-treatment may well have decreased lactate overproduction and ameliorated metabolism-secretion coupling in GK islets.

These metabolic profiles in GK islets resemble the Warburg effect, characteristic aerobic metabolism in cancer cells by which lactate is overproduced from glucose even under non-hypoxic condition (40). Hypoxia-inducible factor 1α (HIF1α) is a transcription factor that functions as a master regulator of oxygen homeostasis and upregulates expression of LDH (41). HIF1α activity is controlled at the level of protein stability. When oxygen is present, two proline residues in the oxygen-dependent degradation domain of HIF1α are hydroxylated by the prolylhydroxylase enzymes (PHD) (42). These hydroxylated motifs allow capture by the von Hippel-Lindau (VHL) protein that forms the recognition component of an E3 ubiquitin ligase complex, which targets the hydroxylated HIF1α for poly ubiquitination and proteasomal degradation (43). In the present study, TE-treatment showed a time-dependent decrease in HIF1α
protein levels in GK islets and 50% reduction compared to that in non-treated GK islets at 12 h culture, which could reduce LDH-A protein in TE-treated GK islets. The Warburg effect is usually thought of as a high rate of glycolysis. Since TE-treatment did not affect glucokianse activity, which determines glycolytic velocity in β cells, alteration in the HIF1α level in the pathophysiological range may not affect glycolytic velocity in β cells. In this respect, lactate overproduction in GK islets differs from the typical Warburg effect.

HIF1α down-regulates expression of GLUT2 and glucokinase and up-regulates pyruvate dehydrogenase 1 (PDK1), which inactivates pyruvate dehydrogenase and suppresses metabolism in the Krebs cycle (44) in islets of von Hippel-Lindau gene (vhl) deficient mouse (45). On the contrary, TE-treatment did not affect glucokinase activity and protein level of PDK1 in GK islets. This discrepancy is not fully understood, but one possibility is that the expression level of HIF1α may be higher than the pathophysiological level in vhl-deficient islets. This is supported by the fact that glucose-induced insulin secretion is impaired severely and almost abolished in vhl-deficient islets. The transcript level of LDH-A in vhl-deficient islets is about 20 folds compared to control islets, which is much higher than that of PDK1 (about 5 folds) (46). This suggests that expression of LDH-A is more readily affected by HIF1α expression than that of PDK1.

Increase in basal HIF1α level in GK islets was not found compared to that in Wistar islets. In contrast, basal levels of LDH-A in GK islets was prominently increased compared to that in Wistar islets, suggesting that regulators other than HIF1α may play an important role in the enhancement of basal LDH-A in GK islets. However, our finding in the present study remains valid, as ROS-dependent regulation of LDH-A by HIF1α, which plays an important role in lactate overproduction and impaired
glucose-induced insulin secretion in GK islets, was established. In addition, involvement of HIF1α in lactate overproduction was shown clearly by the finding that HIF1α inhibitor-treatment restored high glucose-induced insulin secretion and suppressed lactate production in GK islets. ROS inhibits PHD activity and stabilizes HIF1α (47,48). In GK islets, an excess amount of ROS may inhibit PHD activity and stabilize the HIF1α protein. Thus, TE-treatment may recover PHD activity by reduction of the ROS level and decrease the HIF1α protein level to eventually ameliorate metabolism-secretion coupling in GK islets.

In conclusion, we show that 12 h suppression of endogenous ROS including \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) improves impaired metabolism-secretion coupling in GK islets. Our results suggest that lactate overproduction plays an important role in impaired metabolism-secretion coupling in diabetic β-cells, ROS reduction improving mitochondrial metabolism by suppressing lactate overproduction through inhibition of HIF1α stabilization.
Table 1

ip-GTT in GK rats treated with tempol plus ebselen in vivo

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<td></td>
<td>0</td>
<td>15</td>
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<td>60</td>
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<td>Glucose (mg/dl)</td>
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<tr>
<td>Control</td>
<td>145 ± 4</td>
<td>475 ± 6</td>
<td>423 ± 11</td>
<td>340 ± 14</td>
<td>184 ± 7</td>
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<td>TE</td>
<td>144 ± 11</td>
<td>330 ± 41†</td>
<td>359 ± 28*</td>
<td>330 ± 24</td>
<td>200 ± 13</td>
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<td>Insulin (pg/ml)</td>
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<tr>
<td>Control</td>
<td>164 ± 70</td>
<td>n.d.</td>
<td>172 ± 50</td>
<td>n.d.</td>
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<td>TE</td>
<td>205 ± 32</td>
<td>n.d.</td>
<td>400 ± 101*</td>
<td>n.d.</td>
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After the intraperitoneal injection of 50 mg/kg tempol plus 10 mg/kg ebselen (TE) or vehicle alone (control) twice during 15 h fast in GK rats, glucose tolerance test with intraperitoneal injection of glucose (1 g/kg body wt) was performed and plasma glucose and insulin levels were measured. Data are means ± SE of 7 independent experiments. *, P<0.05 vs. corresponding control without tempol plus ebselen (TE). †, P<0.01 vs. corresponding control without TE. n.d., not determined.
Table 2

Insulin secretion from isolated islets in GK rats treated with tempol plus ebselen in vivo

<table>
<thead>
<tr>
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<th>2.8 mmol/l glucose</th>
<th>16.7 mmol/l glucose</th>
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<tr>
<td>Control (ng/30min/islet)</td>
<td>0.40 ± 0.04</td>
<td>0.84 ± 0.06</td>
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<tr>
<td>TE (ng/30min/islet)</td>
<td>0.49 ± 0.04</td>
<td>1.42 ± 0.13*</td>
</tr>
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After the intraperitoneal injection of 50 mg/kg tempol plus 10 mg/kg ebselen (TE) or vehicle alone (control) twice during 15 h fast in GK rats, islets were isolated and insulin secretion in the presence of 2.8 and 16.7 mmol/l of glucose was measured. *, P<0.01 vs. corresponding control without TE. Data are means ± SE of 9 determinations.
TABLE 3

Effect of TE-treatment on glucokinase, hexokinase and mitochondrial glycerol-3-phosphate dehydrogenase activities.

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Wistar rat</th>
<th>GK rat</th>
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<tbody>
<tr>
<td>Hexokinase (whole islet extracts)</td>
<td>control</td>
<td>21.58 ± 3.23</td>
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<tr>
<td></td>
<td>tempol+ebselen</td>
<td>21.63 ± 3.18</td>
</tr>
<tr>
<td>Glucokinase (whole islet extracts)</td>
<td>control</td>
<td>22.79 ± 8.21</td>
</tr>
<tr>
<td></td>
<td>tempol+ebselen</td>
<td>22.90 ± 6.53</td>
</tr>
<tr>
<td>mGPDH (whole islet extracts)</td>
<td>control</td>
<td>9.90 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>tempol+ebselen</td>
<td>10.60 ± 0.75</td>
</tr>
<tr>
<td>mGPDH (mitochondrial fraction)</td>
<td>control</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>tempol+ebselen</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

After islets were cultured with or without TE for 12 hours, extract from whole islets and mitochondrial fractions were obtained. Data are given as the mean ± SE from 5 experiments for glucokinase and hexokinase, 6 experiments for mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) using whole islet extracts and 3 experiments for mGPDH using mitochondrial fraction. *, P < 0.05 vs. Wistar control without TE. †, P < 0.05 vs. control without TE. n.d., not determined.
### TABLE 4

ATP production by mitochondria fraction from islets.

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Wistar rat</th>
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<th>GK rat</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>TE</td>
<td>C</td>
<td>TE</td>
</tr>
<tr>
<td>0.5 mM succinate</td>
<td>2.86 ± 0.42</td>
<td>2.58 ± 0.15</td>
<td>2.43 ± 0.27</td>
<td>2.18 ± 0.21</td>
</tr>
<tr>
<td>0.5 mM succinate + 1 µM antimycin A</td>
<td>0.40 ± 0.03</td>
<td>0.46 ± 0.02</td>
<td>0.36 ± 0.00</td>
<td>0.36 ± 0.00</td>
</tr>
<tr>
<td>1 mM succinate + 1 µM rotenone</td>
<td>3.96 ± 0.48</td>
<td>3.55 ± 0.26</td>
<td>2.92 ± 0.22</td>
<td>2.71 ± 0.48</td>
</tr>
<tr>
<td>1 mM glutamate + 1 mM malate</td>
<td>0.92 ± 0.23</td>
<td>0.86 ± 0.14</td>
<td>0.87 ± 0.13</td>
<td>0.90 ± 0.14</td>
</tr>
<tr>
<td>1 mM pyruvate + 1 mM malate</td>
<td>1.32 ± 0.38</td>
<td>1.17 ± 0.29</td>
<td>1.27 ± 0.22</td>
<td>1.23 ± 0.22</td>
</tr>
<tr>
<td>0.5 mM TMPD + 2 mM ascorbate</td>
<td>5.42 ± 0.41</td>
<td>4.91 ± 0.15</td>
<td>6.27 ± 0.62</td>
<td>5.94 ± 0.54</td>
</tr>
<tr>
<td>1 mM glycerol-3-phosphate</td>
<td>0.92 ± 0.14</td>
<td>3.03 ± 0.17†</td>
<td>0.69 ± 0.05</td>
<td>1.50 ± 0.12†</td>
</tr>
</tbody>
</table>

Islets were cultured with or without TE for 12 h. Mitochondrial suspension was obtained from control (C) or TE-treated islets (TE). Mitochondrial ATP production is indicated as the ratio to ATP production from adenylate kinase to normalize the mass of the intact mitochondria obtained, which was determined from the same sample in parallel incubation. Data are given as the mean ± SE of 3 different experiments. †, $P < 0.01$ vs. control without TE.
FIGURE LEGENDS

Fig. 1. Effects of antioxidant enzyme mimics on ROS production in Wistar (A and C) and GK (B and D) islets. Islets were isolated and cultured without (control; white bars) or with 10 mmol/l tempol (T; hatched bars), 10 µmol/l ebselen (E; gray bars), and T plus E (TE; black bars). Superoxide (O$_2^-$) generation (A and B) was determined by measuring the reduction of NBT using 100 islets, and data were shown as fold increase relative to control. H$_2$O$_2$ generation (C and D) was determined by measuring the peroxidation of homovanillic acid included in the reaction mixtures as a substrate using 150 islets. Data are shown as means ± SE of 3 different experiments. †, $P < 0.01$ vs. control.

Fig. 2. Effects of antioxidant enzyme mimics on insulin secretion (AB) and ATP contents (C). Islets were cultured without or with ebselen (E: gray bars) and tempol plus ebselen (TE: black bars) for 12 h. After cultured islets were washed and preincubated with 2.8 mmol/l glucose for 30 min, islets were incubated for 30 min at 2.8 mmol/l and 16.7 mmol/l glucose and released insulin was measured. Insulin release from Wistar islets (A) and in GK islets (B) are shown. After cultured GK islets were washed and preincubated with 2.8 mmol/l glucose for 30 min, islets were incubated for 30 min at 2.8 mmol/l and 16.7 mmol/l glucose and ATP contents in GK islets were measured (C). Data are expressed as the mean ± SE of 25 (AB) or 20 (C) determinations from 3 (AC) and 4 (B) experiments. *, $P < 0.01$ vs. corresponding 2.8 mmol/l glucose. †, $P < 0.01$ vs. 16.7 mmol/l glucose in control. ‡, $P < 0.05$ vs. 16.7 mmol/l glucose in control.
Fig. 3. Effects of TE-treatment on glucose oxidation in Wistar (A) and GK (B) islets. Islets were cultured without (control; open bars) or with TE (black bars) for 12 h. After cultured islets were preincubated with 2.8 mmol/l glucose for 30 min, glucose oxidation during incubation for 90 min in the presence of 2.8 mmol/l and 16.7 mmol/l glucose was measured in islets. Data are shown as means ± SE of 12 determinations from 3 (A) and 4 (B) experiments. *, $P < 0.01$ vs. corresponding 2.8 mmol/l glucose. †, $P < 0.01$ vs. 16.7 mmol/l glucose in control.

Fig. 4. Effects of TE-treatment on lactate production and HIF1α signaling. ACD: Islets were cultured without (control; open bars) or with TE (black bars) for 12 h. A: Effects of TE-treatment on lactate production in Wistar and GK islets. After cultured islets were preincubated under 2.8 mmol/l glucose for 30 min, 20 islets were batch incubated in the medium containing 2.8 and 16.7 mmol/l glucose for 60 min at 37ºC and released lactate in the medium was measured. Values are means ± SE of 13 determinations from 3 (Wistar) and 4 (GK) experiments. B: Time course of HIF1α protein levels in GK and Wistar islets during TE-treatment using whole islet lysates. Islets were cultured without (control; open bars) or with TE (closed bars) for indicated hours (H). The bar graphs are expressed relative to 3 h-cultured control values corrected by β-actin level. The values are means ± SE of determinations from three different experiments. Representative blot panels are shown. C: Effect of TE-treatment on protein levels of HIF1α and HIF1α downstream target proteins in whole lysate of Wistar and GK islets. Representative blot (left) and quantification data (right) are shown. The bar graphs are expressed relative to Wistar without TE-treatment values corrected by β-actin level. The values are means ± SE of determinations from 3 different experiments. *, $P < 0.01$ vs. corresponding 2.8 mmol/l glucose. †, $P < 0.01$ vs. corresponding control without TE. ‡, $P < 0.01$ vs.
corresponding Wistar.

Fig. 5 Effects of HIF1α inhibitor on insulin secretion and lactate production in GK islets. Islets were cultured without (control; open bars) or with 1 µmol/l HIF1α inhibitor (black bars) for 12 h. A. Insulin secretion. After cultured islets were preincubated with 2.8 mmol/l glucose for 30 min, insulin secretion during incubation for 30 min in the presence of 2.8 and 16.7 mmol/l glucose was measured in islets. Data are shown as means ± SE of 25 determinations from 3 experiments. *, \( P < 0.01 \) vs. corresponding 2.8 mmol/l glucose. †, \( P < 0.01 \) vs. 16.7 mmol/l glucose in control. B. Lactate production. After cultured islets were preincubated under 2.8 mmol/l glucose for 30 min, islets were batch incubated in the medium containing 2.8 and 16.7 mmol/l glucose for 60 min and released lactate in the medium was measured. Values are means ± SE of 12 determinations from 3 experiments. *, \( P < 0.01 \) vs. corresponding 2.8 mmol/l glucose. ‡, \( P < 0.01 \) vs. corresponding control without HIF1α inhibitor.
Acknowledgments

This study was supported by a Research Grant on Nanotechnical Medicine from the Ministry of Health, Labour, and Welfare of Japan, Scientific Research Grants, a grant from Innovation Cluster Kansai project of the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a grant from CREST of Japan Science and Technology Cooperation.

No potential conflicts of interest relevant to this article were reported.

Author Contributions: M.S. researched data, contributed to the discussion, wrote the manuscript, and reviewed/edited the manuscript. S.F. contributed to the discussion, wrote the manuscript, and reviewed/edited the manuscript. Y.N., E.M., G.Y., Y.S., H.S, Y.T., and K.O. researched data. K.N. and N.I. contributed to the discussion and reviewed/edited the manuscript. S.F. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

We thank C. Kotake and M. Akazawa (Department of Diabetes and Clinical Nutrition, Graduate School of Medicine, Kyoto University, Japan) for technical assistance.

Parts of this study were presented in abstract form at the 72nd Scientific Sessions of the American Diabetes Association, Philadelphia, PA, June 8-12, 2012.
References


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β-cell function. Diabetes 58:433-441, 2009


Fig. 1

A

NBT reduction (%)

C T E TE

B

NBT reduction (%)

C T E TE

C

H₂O₂ (nmol/30min/150 islets)

C T E TE

D

H₂O₂ (nmol/30min/150 islets)

C T E TE

†
Fig. 2

A

![Bar chart showing insulin release (ng/30 min/islet) at different glucose concentrations (2.8 and 16.7 mM) for conditions C, E, and TE.](image)

B

![Bar chart showing insulin release (ng/30 min/islet) at different glucose concentrations (2.8 and 16.7 mM) for conditions C, E, and TE.](image)

C

![Bar chart showing ATP content (pmol/islet) at different glucose concentrations (2.8 and 16.7 mM) for conditions C and TE.](image)
Fig. 3

A

Glucose oxidation (pmol / islet / 90min)

Glucose (mM) 2.8 16.7 2.8 16.7
C TE

B

Glucose oxidation (pmol / islet / 90min)

Glucose (mM) 2.8 16.7 2.8 16.7
C TE
Fig. 4

A

![Graph showing lactate production in islets from Wistar and GK rats with different glucose concentrations (2.8 and 16.7 mM). The graph includes error bars indicating variability. Asterisks (*) and daggers (†) denote statistical significance.](image)

B

![Image of Western blot for HIF1α protein expression with intensity ratios at 3, 6, 9, and 12 hours. The graph includes error bars indicating variability. Daggers (†) denote statistical significance.](image)
Fig. 4

C

HIF1α

LDH-A

PDK1

β-actin

Intensity (ratio)

Intensity (ratio)

Intensity (ratio)

C  TE  C  TE

Wistar  GK
Fig. 5

A

Insulin release (ng/30min/islet)

Glucose (mM) 2.8 16.7 2.8 16.7

* C HIF1α inhibitor

B

Lactate (pmol/islet)

Glucose (mM) 2.8 16.7 2.8 16.7

* HIF1α inhibitor

‡ * †
LEGENDS of SUPPLEMENTARY FIGURES

Supplementary Fig. 1. Effects of TE-treatment on protein expression levels of UCP2 and mitochondrial respiratory chain proteins in Wistar and GK islets. Islets were cultured without (control; open bars) or with TE (black bars) for 12 h. Protein expression was analyzed using immunoblot analysis. UCP2 protein level (A) was determined using mitochondrial fraction from cultured islets, and mitochondrial respiratory chain protein levels (B) were determined using whole-islets lysates. Intensities of the bands were quantified with densitometric imager. The bar graphs are expressed relative to Wistar control values corrected by Hsp-60 level in UCP2 and by β-actin level in mitochondrial respiratory chain proteins, respectively. Data are expressed as means ± SE of determinations relative to from 3 different experiments. Representative blot panels are shown.

Supplementary Fig. 2. HIF1α protein levels in INS-1 cells during CoCl2 treatment. INS-1 cells were cultured with 200 µmol/l CoCl2 for indicated hours (H). Protein levels of HIF1α were analyzed by immunoblot analysis using whole cell lysates.

Supplementary Fig. 3. Effect of TE-treatment on protein levels of M2-pyruvate kinase (PK) in whole lysate of GK islets. Representative blot (left) and quantification data (right) are shown. The bar graphs are expressed relative to control without TE-treatment values corrected by β-actin level. The values are means ± SE of determinations from 3 different experiments. *, P < 0.01 vs. control without TE.
Supplementary Fig. 1

A

UCP2
HSP-60

Intensity (ratio)

C  TE  C  TE
Wistar  GK

B

Complex I
β-Actin
Complex III
β-Actin
Complex IV
β-Actin
Complex V
β-Actin

C  TE  C  TE
Wistar  GK
Supplementary Fig. 2

**INS-1 cell**

HIF1α

0H 4H 8H 16H 20H

200μmol/l CoCl₂

Supplementary Fig. 3

**M2-PK**

β-actin

C TE

GK

<table>
<thead>
<tr>
<th>C</th>
<th>TE</th>
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<td></td>
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