Pteridines
Vol. 19, 2008, pp. 93 - 100

Insulin Secretion in Response to L-Arginine under Decreasing Tetrahydrobiopterin Content

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Abstract

Tetrahydrobiopterin (BH_4) content in the pancreas from GK (Goto-Kakizaki) rats as a type II diabetic model was markedly decreased compared to Wistar rats as a healthy control. Moreover, insulin stimulated BH_4 synthesis in a hamster pancreatic β-cell line, HIT-T15 cells. Therefore, in diabetic condition, the reduction of insulin action is likely to lead to the low levels of BH_4 content in pancreatic β-cells. In this study, we examined the effects of the decrease in BH_4 content on insulin secretion by L-arginine (L-Arg) or glucose using HIT-T15 cells. Cytosolic calcium concentration ($[Ca^{2+}]_i$) in HIT-T15 cells was increased by L-Arg. Under the low BH_4 content using 2,4-diamino-6-hydroxypyrimidine (DAHP), a selective inhibitor of BH_4 synthesis, L-Arg-induced both Ca^{2+} response and insulin secretion were stimulated. These stimulating effects were recovered by BH_4 content recovering, using sepiapterin, a precursor of BH_4 biosynthesis. By contrast, glucose-induced Ca^{2+} response was not affected by the BH_4 content. These results suggest that the decrease of BH_4 content in β-cells may stimulate insulin secretion by L-Arg, but not by glucose, under diabetic conditions.

Key words: tetrahydrobiopterin, nitric oxide, calcium, L-arginine, insulin, β-cell

Introduction

Tetrahydrobiopterin (BH_4), one of the essential cofactors of nitric oxide (NO) synthase, is decreased in insulin-resistant rat aorta (1). Moreover, in streptozotocin-induced diabetic rat brain, BH_4 content was also reduced (2). We previously reported that insulin stimulates BH_4 synthesis in brain microvascular endothelial cells (3). Therefore, in diabetic condition, the reduction of insulin action may lead to the low levels of BH_4 content in many types of tissues including pancreas. In this study, we first measured BH_4 content of pancreas in non-insulin dependent rat (GK rat), and found the reduction of it.

In our previous study, the reduction of BH_4 content stimulated the increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) by L-Arg (4). In addition, the increase of Ca^{2+} response is essential for the beginning of insulin secretion from β-cells by numerous insulinotropic substances including amino acid (5-9). Thus, under low concentration of BH_4, insulin secretion in response to L-Arg is likely to be stimulated by the increase of Ca^{2+} response. In this study, we examined the effect of low concentration of BH_4 in L-Arg-induced either Ca^{2+} response or insulin secretion using HIT-T15 cells, hamster pancreatic β-cell line. Moreover, the effect of glucose on insulin secretion at low BH_4 levels was also examined.

Material and Methods

Experimental animals

Spontaneously diabetic Goto-Kakizaki (GK, 235-290 g) rats and non-diabetic Wistar rats (330-394 g) were obtained from Saitama Experimental Animal Supply. Inc. (Saitama, Japan). All animal experiments were approved by the Experimental Animal Committee of the School of Pharmaceutical Sciences, Showa University.

Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). The pancreas was dissected from rats and homogenized in ice-cold Tris buffer (pH 7.4) containing 1 mM dithiothreitol, 1 mM EDTA and 10 mM Tris for BH_4 assay sample. Serum was obtained by centrifugation of blood at 2500 rpm for 15 min at 4°C,

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and serum glucose level was determined using a glucose oxidase kit (Glucose CII-Test Wako).

Cell culture

The hamster pancreatic β-cell line, HIT-T15 cells (Dainippon Pharmaceutical Co., Osaka, Japan; passages 70-90) were cultured in Ham'F12 medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. PC12 cells were donated by the Japanese Cancer Research Resources Bank (Tokyo, Japan), and were cultured in RPMI 1640 medium supplemented with heat-inactivated 5% horse serum, 5% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Measurement of biopterin derivatives

Biopterin was quantified by reversed-phase high performance liquid chromatography (HPLC) with fluorometric detection, as previously described by Fukushima and Nixon (10). The amount of BH_4 was calculated from the difference in the biopterin concentrations measured after oxidation in the acid (total biopterins) and the base (biopterin + 7,8-dihydrobiopterin (BH_2)). Intracellular BH_4 concentration was reduced by DAHP and recovered by sepiapterin, as previously described by Gross and coworkers (11).

Measurement of changes in $[Ca^{2+}]_i$

Cells on coverslips were loaded with fura-2 in HEPES-buffered saline (HBS) containing (in mM) 107 NaCl, 6 KCl, 1.2 $MgSO_4$, $2CaCl_2$, 20 HEPES, adjusted to pH 7.4 with NaOH. Cells were incubated with HBS containing 5 μM fura-2/AM (Dojindo Laboratories, Kumamoto, Japan) and 0.1% bovine serum albumin at 37°C for 40 min. The fura-2-loaded cells were then incubated with HBS for 20 min. The coverslips were then placed in a perfusion chamber mounted on the stage of the microscope. Fluorescence images of the cells were recorded and analyzed with a video images analysis system (MetaFluor, Nippon Roper, Osaka, Japan). Fluorescence emission at 510 nm was monitored with excitation at 340 nm and 380 nm. The pairs of 340 nm and 380 nm fluorescence images were captured every 5 sec. The 340/380 nm ratio was calculated after subtraction of background fluorescence. Data from all measurements are expressed as fluorescence ratio of 340/380 nm.

Detection of neuronal NO synthase mRNA by RT-PCR

Total RNA was extracted by a modified guanidinium isothiocyanate method using TRIzol Reagent (Invitrogen Co., Ltd., Carlsbad, CA, USA). Reverse transcription and PCR amplification from 0.5 μg of total RNA were performed using r*Tth* DNA polymerase (RT-PCR high Plus, Toyobo Co., Tokyo, Japan). The pair of primers used for amplification of neuronal NO synthase (NM_008712) sequences was 5'-GACCCATGTGGTCCTCATTC-3' and 5'-CCTGGATTCCTGTGTCTTTC-3'. The thermocycler was programmed to give an initial cycle consisting of reverse transcription at 60°C for 30 min, and denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min and annealing/extension at 62°C for 1.5 min. To control for the amounts of total RNA, parallel RT-PCR of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was performed as a reference, using the pair of primers 5'-TCCACCACCCTGTTGCTGTA-3' and 5'-ACCACAGTCCATGCCATCAC-3'. PCR products were electrophoresed on a 3% Agarose S (Wako Pure Chemicals, Osaka, Japan) gel containing ethidium bromide and visualized by UV-induced fluorescence.

Measurement of insulin secretion

The cells were incubated for 60 min with HBS, and then treated with HBS including L-Arg (2.5 mM) for 60 min. The amount of insulin in the culture supernatant was determined with an EIA kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA).

Materials

L-Arginine hydrochloride and nifedipine were from Wako Pure Chemicals (Osaka, Japan). 2,4-Diamino-6-hydroxypyrimidine (DAHP) was from Aldrich Chemical Co. (Tokyo, Japan). Verapamil was from Sigma Chemical Co. (St. Louis, MO). Sepiapterin was from Funakoshi (Tokyo, Japan). All other reagents were of the highest grade commercially available.

Statistical analysis

Data are presented as means ± S.E.M.. The statistical significance of observed differences was determined by analysis of variance followed by Bonferroni's method. Differences between means were considered significant when P was less than 0.05.

Results

BH_4 content in pancreas from Wistar and GK rats

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The biopterin derivatives levels (BH_4, BH_2 and biopterin) in the pancreas of Wistar and GK rats were measured. The biopterin derivatives content, especially BH_4 content, was significantly decreased in the pancreas of GK rats compared to control rats (Wistar rats) (Figure 1).

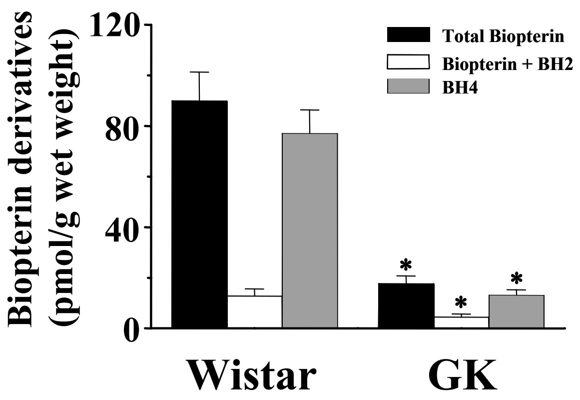

Figure 1: Biopterin derivatives content in the pancreas from Wistar and GK rats. Spontaneously diabetic GK rats (body weight: 235-290 g, plasma glucose levels: 216-308 mg/dl, n=5) and non-diabetic Wistar rats (body weight: 330-394 g, plasma glucose levels : 100-114 mg/dl, n=6) were used. Samples were separately oxidized under acid and alkaline conditions, and BH_4, BH_2 plus biopterin, and total biopterin were determined. Results are the means ± S.E.M. of 5 or 6 rats. *Significant changes in comparison to the Wistar rat group ($p<0.05$).

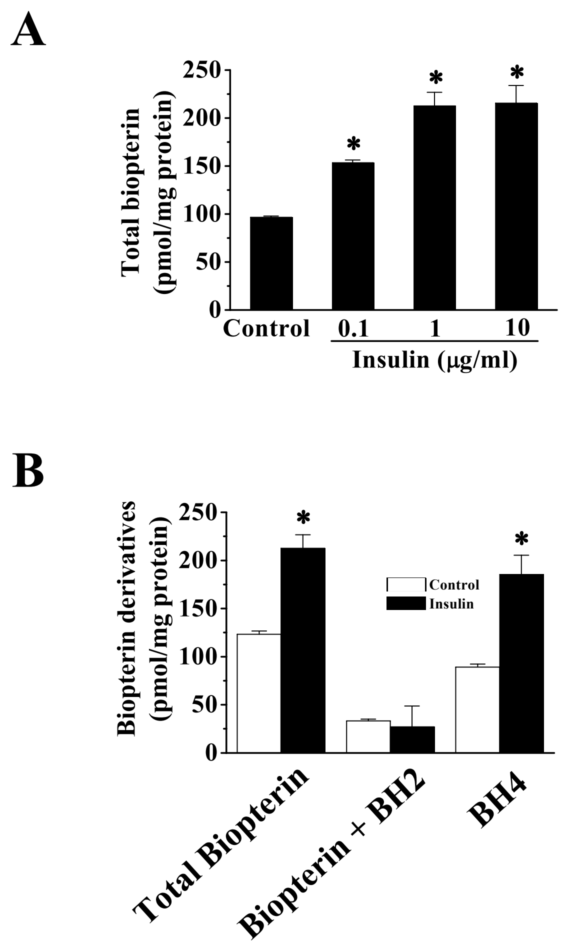

Figure 2: Effect of insulin on biopterin derivatives content in HIT-T15 cells. In A, cells were treated with insulin (0.1-10 μg/ml) for 12 h at 37°C. Total biopterin (BH_4 plus more oxidized forms) content was determined. In B, cells were incubated with or without 1 μg/ml insulin for 12 h. Samples were separately oxidized under acid and alkaline conditions, and BH_4, BH_2 plus biopterin, and total biopterin were determined. Results are the means ± S.E.M. of 4 assays. *Significant changes in comparison to control group ($p<0.05$).

Effect of insulin on BH_4 content in HIT-T15 cells

We examined the effects of insulin on total biopterin contents (BH_4 plus BH_2 plus biopterin) in HIT-T15 cells and observed significant increases of total biopterin by treatment with insulin (0.1-10 μg/ml) for 12 h in a concentration-dependent manner (Figure 2A). As shown in Figure 2B, in contrast to oxidized forms, insulin (1 μg/ml) markedly increased BH_4 content.

Effects of BH_4 and NO on L-Arg-induced Ca^{2+} elevation and insulin secretion

In the next step, we examined whether L-Arg, the substrate of NO synthase, induces Ca^{2+} response in HIT-T15 cells. The addition of L-Arg (10 mM) to HIT-T15 cells increased $[Ca^{2+}]_i$ dependent on the presence of glucose (Figure 3A). In the presence of 11.5 mM glucose, L-Arg increased $[Ca^{2+}]$i in a concentration-dependent manner (Figure 3B and 3C). The optical isomer D-Arg (10 mM), which is not a substrate of NO synthase, did not affect the $[Ca^{2+}]_i$ (Figure 3B and 3C). Moreover, blockers of voltage-operated L-type Ca^{2+} channel, nifedipine (10 μM) and verapamil (10 μM), reduced L-Arg (2.5 mM)-induced $[Ca^{2+}]_i$ elevation (data not shown), suggesting that the L-Arg-induced Ca^{2+} response is dependent on Ca^{2+} influx through the L-type Ca^{2+} channel.

We examined the effects of intracellular BH_4 content on L-Arg-induced Ca^{2+} response. Our previous report suggested that the decrease of intracellular biopterin content by treatment with DAHP (1 mM), a selective inhibitor of GTP cyclohydrolase I (GTPCH), which is late-limiting enzyme of BH_4 synthesis, was recovered to control levels when co-treated with sepipaterin (10 μM), a precursor of BH_4 biosynthesis (11). As shown in Figure 3D, L-Arg (2.5 mM)-induced $[Ca^{2+}]_i$ elevation was stimulated in DAHP (1 mM)-treated cells, and the stimulation response was reduced by co-treatment with sepiapterin (10 μM). In DAHP (0.1-10 mM)-pretreated cells, L-Arg (2.5 mM)-induced $[Ca^{2+}]_i$ elevation was concentration-dependently increased (Figure 3E). Moreover, the stimulation

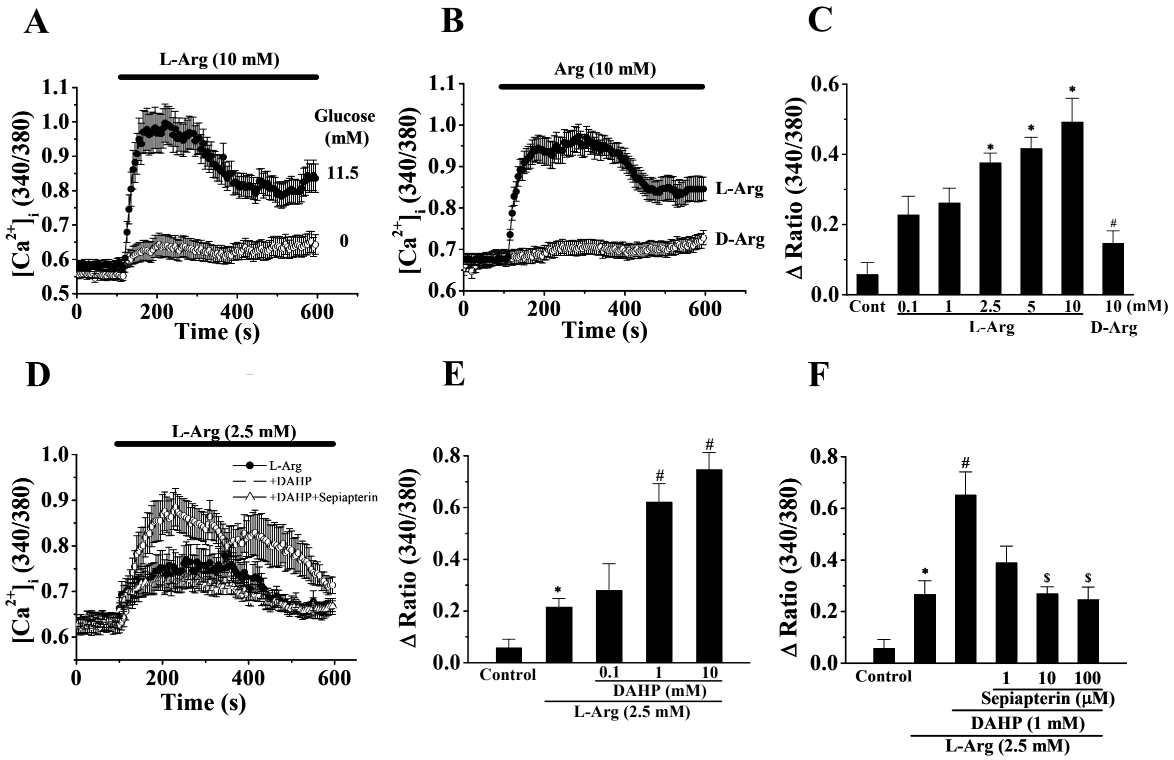

Figure 3: Effect of endogenous BH_4 on L-Arg-induced Ca^{2+} response in HIT-T15 cells. In A, cells were treated with L-Arg (10 mM) in the absence or presence of glucose (11.5 mM). In B and C, cells were treated with L-Arg (0.1-10 mM) or D-Arg (10 mM) in the presence of glucose (11.5 mM). In D, E and F, cells were pretreated with DAHP (D and F: 1 mM, E: 0.1-10 mM) and/or sepiapterin (D: 10 μM, F: 1-100 μM) for 6 h, and then cells were treated with L-Arg (2.5 mM). The effects of DAHP and sepiapterin on peak levels of L-Arg (2.5 mM)-induced $[Ca^{2+}]_i$ elevation was measured. Results are the means ± S.E.M. of 38-173 cells. *Significant changes in comparison to control group ($p<0.05$). #Significant changes in comparison to L-Arg (10 mM or 2.5 mM)-treated group ($p<0.05$). $Significant changes in comparison to DAHP (1 mM)-pretreated group ($p<0.05$).

of L-Arg-induced Ca^{2+} response in DAHP (1 mM)-treated cells was reduced by co-treatment with sepiapterin (1-100 μM) in a concentration-dependent manner (Figure 3F).

To determine whether L-Arg (2.5 mM)-induced Ca^{2+} response is regulated by NO, the effects of carboxy-PTIO, a scavenger of NO, and SNAP, a NO donor, were examined. As shown in Figure 4A, we observed the expression of neuronal NO synthase mRNA in HIT-T15 cells. The L-Arg-induced Ca^{2+} response was stimulated in carboxy-PTIO-treated cells, and the stimulation response was reduced by treatment with SNAP (10 nM, Figure 4B). Moreover, the acceleration of L-Arg-induced $[Ca^{2+}]_i$ elevation in DAHP-treated cells was also reduced by treatment with SNAP (10 nM, Figure 4C).

As shown in Figure 5, L-Arg (2.5 mM) stimulated insulin secretion. L-Arg-induced insulin secretion was stimulated in carboxy-PTIO-treated cells, and the stimulation response was reduced by treatment with SNAP (10 nM, Figure 5A). L-Arg-induced insulin secretion was also promoted by pretreatment with DAHP (1 mM, Figure 5B). Moreover, the acceleration of L-Arg (2.5 mM)-induced insulin secretion in DAHP-pretreated cells was also reduced by treatment with SNAP (10 nM) or by co-treatment with sepiapterin (10 μM).

Effects of BH_4 and NO on glucose-induced Ca^{2+} elevation

The addition of glucose (11.5 mM) to HIT-T15 cells increased $[Ca^{2+}]_i$ (Figure 6A). To determine whether glucose-induced Ca^{2+} response is regulated by intracellular BH_4 content and/or NO, the effects of DAHP (1 mM) and carboxy-PTIO (10 μM) were examined. As shown in Figure 6B, neither DAHP nor carboxy-PTIO affected the glucose-induced Ca^{2+} response.

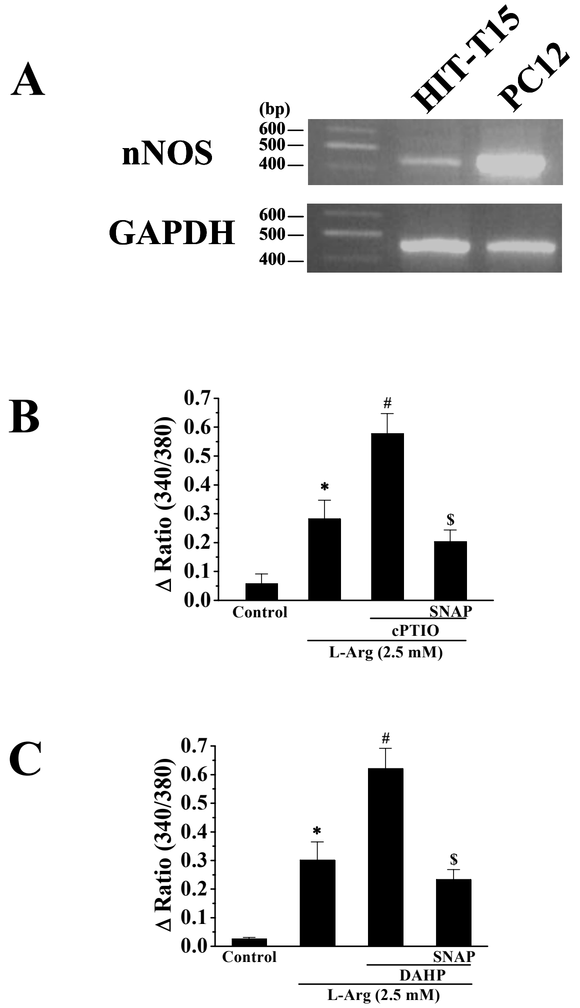

Figure 4: Effect of NO on L-Arg-induced $[Ca^{2+}]_i$ elevation in HIT-T15 cells. In A, neuronal NO synthase (nNOS) mRNA levels in HIT-T15 and PC12 cells were determined by RT-PCR. In B, cells were pretreated with carboxy-PTIO (cPTIO, 10 μM) for 3 min, and then treated with L-Arg (2.5 mM) and/or SNAP (10 nM). In C, cells were pretreated with DAHP (1 mM) for 6 h, and then treated with L-Arg (2.5 mM) and/or SNAP (10 nM). Results are the means ± S.E.M. of 22-116 cells. *Significant changes in comparison to control group ($p<0.05$). #Significant changes in comparison to L-Arg (2.5 mM)-treated group ($p<0.05$). $Significant changes in comparison to DAHP- or cPTIO-pretreated groups ($p<0.05$).

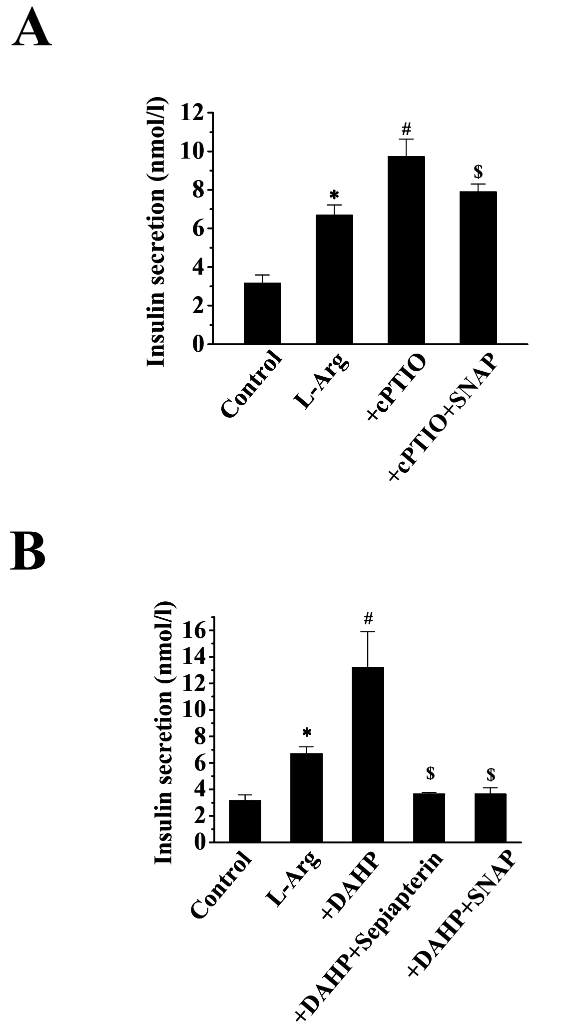

Figure 5: Effects of BH_4 and NO on L-Arg-induced insulin secretion in HIT-T15 cells. In A, cells were pretreated with carboxy-PTIO (cPTIO, 10 μM) for 3 min, and then treated with L-Arg (2.5 mM) and/or SNAP (10 nM). In B, cells were pretreated with DAHP (1 mM) and/or sepiapterin (10 μM) for 6 h, and then treated with L-Arg (2.5 mM) and/or SNAP (10 nM) in HBS without DAHP and sepiapterin. Insulin secretion was determined by EIA assay. Results are the means ± S.E.M. of 3-4 assays. *Significant changes in comparison to control group ($p<0.05$). #Significant changes in comparison to L-Arg (2.5 mM)-treated group ($p<0.05$). $Significant changes in comparison to DAHP- or cPTIO-pretreated groups ($p<0.05$).

Discussion

It is known that BH_4 content is decreased in insulin-resistant rat aorta (1) and in streptozotocin-induced diabetic rat brain (2). Our results suggest that BH_4 content of the pancreas in the type II diabetic mellitus model rat (GK rat) is also significantly decreased, in comparison to healthy rats. On the other hand, insulin stimulates not only the BH_4 synthesis pathway in vascular endothelial cells and the aorta (1,3,12), but also the BH_4 recycle system in adrenal medulla and cortex

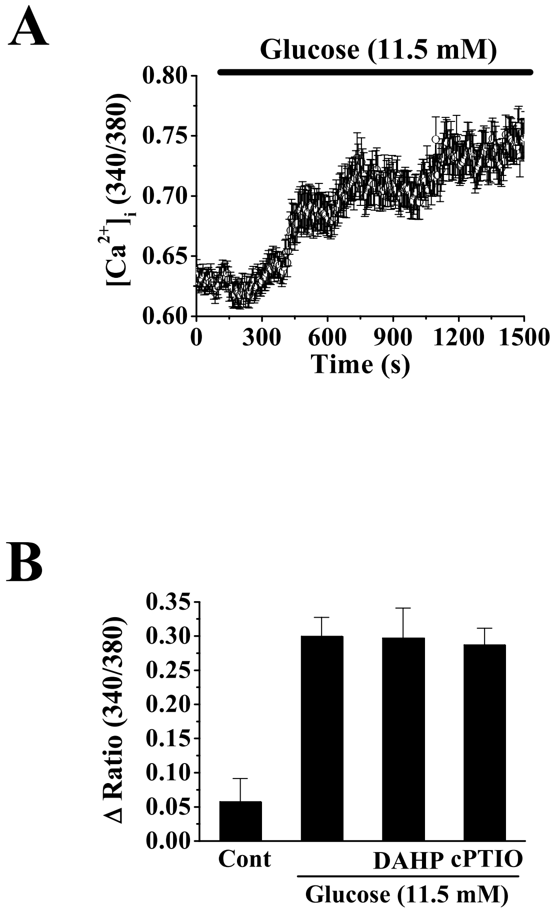

Figure 6: Effects of DAHP and carboxy-PTIO on glucose-induced $[Ca^{2+}]_i$ elevation in HIT-T15 cells. In A, cells were incubated with fura-2 in HBS excluding glucose for 40 min. Fura-2-loaded cells were then incubated with HBS excluding glucose for 20 min. Glucose (11.5 mM)-induced $[Ca^{2+}]_i$ changes were expressed as the fura-2 fluorescence ratio (340/380 nm). In B, cells were pretreated with DAHP (1 mM) for 6 h or carboxy-PTIO (cPTIO, 10 μM) for 3 min, and then treated with glucose (11.5 mM). Results are the means ± S.E.M. of 40-107 cells.

(13). In addition, we also observed that insulin stimulates BH_4 synthesis in pancreatic β-cell line HIT-T15 cells. Luciani and Johnson (14) reported that autocrine insulin signaling directly controls insulin protein levels. Therefore, the reduction of autocrine insulin signaling under diabetic condition seems to decrease BH_4 synthesis in β-cells.

It is well known that $[Ca^{2+}]_i$ elevation triggers insulin secretion in pancreatic β-cells (5-8, 15,16). It has been shown that cationic amino acids, including L-Arg, directly depolarize the plasma membrane, and as a consequence, open L-type Ca^{2+} channels (17). On the other hand, it is known that NO, which is known to be produced by NO synthase from L-Arg, inhibits L-type Ca^{2+} current (18,19), and suppresses insulin secretion (20,21). Pancreatic β-cells constitutively express neuronal NO synthase, one of the constitutive NO synthases, which is activated by Ca^{2+} (9,22,23). We also confirmed the expression of neuronal NO synthase mRNA in HIT-T15 cells. In the present study, we demonstrate that L-Arg-induced both Ca^{2+} response and insulin secretion were stimulated by not only NO scavenging but also by reduction of the BH_4 content, and these stimulating responses were reduced by co-treatment with an NO donor. Therefore, the reduction of BH_4 content may lead to stimulate insulin secretion through the decrease of NO production from NO synthase; however, the relation between Ca^{2+} response and NO production after treatment with L-Arg in HIT-T15 cells was not clear. Future studies are needed to determine the relation between Ca^{2+} response and NO production in L-Arg-treated HIT-T15 cells.

Under diabetic conditions, the ability of glucose-sensitive insulin secretion was decreased (24). Interestingly, L-Arg-evoked Ca^{2+} response was maintained in diabetic β-cells (24). Zaitsev et al. (25) reported that the L-Arg-induced Ca^{2+} response is more active in islets from the type II diabetic mellitus model rats than in islets from normal rats. In this study, although the decrease in BH_4 content stimulated L-Arg-induced Ca^{2+} response, it had no effect on glucose-induced Ca^{2+} response. Thus, it is possible that the decrease of BH_4 content in diabetic β-cells may be one reason for the preservation of L-Arg-induced Ca^{2+} responses. On the other hand, the decrease of BH_4 content is not a reason for the reduction of glucose-induced Ca^{2+} response in diabetic β-cells.

It is widely accepted that β-cells have little protection ability against reactive oxygen species (ROS), including H_2O_2, since β-cells have a low expression of antioxidant enzymes (26-29). Under diabetic conditions, ROS were produced and involved in the progression of β-cell dysfunction. BH_4 has scavenging activity for ROS and antioxidative activity (30-32). In addition, BH_4 suppresses ROS production from neuronal NO synthase (33). Thus, BH_4 may act not only as a negative regulator of ROS production from NO synthase but also as a scavenger of ROS, and may protect β-cells against ROS. Experiments with antioxidant compounds other than BH_4 would be able to demonstrate to what extent they may be able to manipulate, e.g., insulin secretion by L-Arg. Unfortunately, such data are not available yet. However, from the data of this study we can speculate that the decrease in BH_4 content of β-cells may stimulate L-Arg-evoked insulin secretion as part of the cell defense system, since insulin stimulates BH_4 synthesis.

In conclusion, the reduction of insulin secreting ability in GK rat is likely to be involved in low BH_4 content in pancreas, since insulin has the stimulating effects of BH_4 synthesis in several cells including β-cells. Moreover, under low levels of BH_4, like diabetic condition, not only Ca^{2+} elevation but also insulin secretion in response to L-Arg was stimulated. However, the reduction of endogenous BH_4 content did not regulate glucose-induced insulin secretion. In the future study, we will need to examine the role of insulin secretion in response to L-Arg under diabetic mellitus.

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