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Original Article

Putrescine and Cadaverine Enhance Insulin Secretion of Mouse Pancreatic β-cell Line

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We examined the effects of polyamines such as putrescine and cadaverine on the biosynthesis and secretion of insulin in the mouse pancreatic β-cell line, MIN-6. Basal insulin secretion (BIS) and glucose-stimulated insulin secretion (GSIS) from the MIN-6 cells were significantly increased by 20 min- or 24 h-treatment with micromolar concentrations of polyamines. To determine whether the enhancement was due to increase of insulin production by polyamines, we investigated the insulin mRNA and protein production. Both insulin mRNA and protein production were found to be not significantly affected by the polyamine treatment. Next, we examined the expression of several transcription factors (TFs) related to insulin synthesis and secretion in order to identify upstream events responsible for the promotion of insulin secretion of MIN6 cells by polyamines. Of the 6 TFs tested, MafA was induced by treatment of polyamines. MafA mRNA and protein expressions increased with treatment of polyamines. Overall results suggest that cadaverine and putrescine promote the insulin secretion process rather than the insulin biosynthesis from MIN6 cells. Also MafA may be involved in the enhanced insulin secretion process. Further studies are needed to elucidate the underlying mechanisms for promotion of insulin secretion by polyamines.

Key Words: Putrescine, Cadaverine, MIN-6, Pancreatic β-cell, Insulin secretion

INTRODUCTION

Polyamines are biological compounds consisting of a carbon chain with 2, 3 or 4 amino groups, which appear to have a positive charge in physiological pH (Janne et al., 2005). Representative polyamines include putrescine, spermidine, and spermine (Pegg, 1988). Polyamines are known to be involved in the regulation of cellular proliferation and differentiation (Pegg, 1988) and they exist in pancreatic β-cells in high concentrations (Hougaard et al., 1986). Previous studies have demonstrated that intracellular polyamines act as stimulatory factors for insulin production and secretion (Welsh and Sjoholm, 1988; Sjoholm et al., 1990; Welsh, 1990). These studies have used inhibitors that directly or indirectly block intracellular polyamine biosynthesis, and thus they did not evaluate the effects of exogenous polyamines on insulin production and secretion. In another study, exogenous treatment of 10 mM spermine was found to suppress insulin secretion through inhibition of protein kinase C (Thams et al., 1986). In contrast, a recent study has revealed that treatment by relatively low concentrations of 100 μM spermidine enhances insulin secretion, while spermine at the same concentration has no effect (Ohtani et al., 2009). Therefore, the role of exogenous polyamines on insulin secretion remains uncertain.

In this study, we wanted to determine the role of exogenous polyamines, such as cadaverine and putrescine, in insulin production and secretion of pancreatic β-cell line, MIN6. To achieve this, we examined basal insulin secretion (BIS), glucose-stimulated insulin secretion (GSIS), and expression of several transcription factors associated with

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insulin production and secretion.

MATERIALS AND METHODS

Chemicals and cells

Putrescine and cadaverine were purchased form Sigma Aldrich Corp. (St. Louis, Mo, US). Fetal bovine serum (FBS) was obtained from Lonza Ltd. (Basel, Switzerland). Mouse pancreatic beta-cell line, MIN6, was a gift from Prof. HS Jeon (Lee Ghil Ya Cancer & Diabetes Institute) and was routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, CA, USA) containing 25 mM glucose, 10% heat-inactivated FBS, 1% Antibiotic-Antimycotic (Invitrogen Corp., Gibco BRL, MD, USA) at 37℃ in a 5% CO_2 humidified incubator.

Cell viability analysis

Cell viability and proliferation were determined with Ez-Cytox Cell Viability Assay Kit (Daeil Lab Service Co., Seoul, Korea) based on the cleavage of the tetrazolium salt to water-soluble formazan by succinate-tetrazolium reductase. Briefly, cells were treated with various concentrations (1~100 μM) of putrescine or cadaverine for 24 h. After treatment, cells were transferred into 96 well plates in 100 μl of medium and incubated with 10 μl of Ez-Cytox solution for 6 h in the 37℃ incubator. Then, absorbance was measured using the ELISA Reader (μ-Quant, Bio-Tek Instruments, Winooski, VT, US) at 450 nm.

Measurement of insulin production and secretion

MIN6 cells were in a 96-well plate at 2.5 × 10^4 cells/well and then cultured for 48 h in supplemented DMEM. The cells were washed once with HEPES-balanced Krebs-Ringer bicarbonate buffer (KRBB) containing 2.8 mM glucose and 0.1% fatty acid-free BSA, and pre-incubated for 1 h at 37℃. After pre-incubation, the cells were treated with putrescine or cadaverine in HEPES-balanced KRBB (pH 7.4) containing 2.8 mM (for BIS) or 16.8 mM glucose (for GSIS) at 37℃ for 20 min. In case of 24 h-treatment, cells were treated with putrescine or cadaverine in supplemented DMEM. After 24 h, the cells were washed once with HEPES-KRBB containing 2.8 mM glucose and 0.1% fatty acid-free BSA, and incubated for 1 h at 37℃. After incubation, the cells were treated with 2.8 or 16.8 mM glucose in HEPES-balanced KRBB (pH 7.4) at 37℃ for 20 min. Insulin secreted into the supernatant was measured using mouse insulin ELISA kits according to the manufacturer's instructions. To measure intracellular insulin content, cells were washed once with phosphate-buffered saline, and acid ethanol solution (75% ethanol, 0.2 mM HCl) was added to each well, followed by overnight incubation at -4℃. After incubation, supernatants were collected by centrifugation at 700 x g for 3 min and insulin levels were measured by ELISA.

RT-PCR

To analyze mRNA expressions of insulin and several transcription factors, MIN6 (7 × 10^4 cells/ml) were seeded on a 24 well plate and were treated with varying concentrations of cadaverine or putrescine (0~100 μM) for 20 min or 24 h. Cadaverine/putrescine-treated or -untreated MIN6 cells were harvested and washed with PBS. Total RNA was isolated using the Qiagen RNAeasy kit according to the manufacturer's instructions (QIAGEN, Valencia, CA, USA). The concentration and purity of the RNA were determined by OD260/280 reading. The cDNA was synthesized from 2 μg of total RNA by use of Reverse Transcriptase M-MLV (Bioprince, Atlanta, GA, US) using an Oligo (dT) primer (Invitrogen) at 50℃ for 1 h. PCR amplification was performed using following primer sets: Insulin1 5'-cagctataatcagagaccat-3', 5'-cagtagttctccagctggta-3', Insulin2 5'-ccctaagtgatccgctacaa-3', 5'-attcattgcagaggggtagg-3', GAPDH 5'-gaggggccatccacagtcttc-3', 5'-catcaccatcttccaggagcg-3', MafA 5'-atcatcact ctgcccaccat-3', 5'-ccgccaacttctcgtatttc-3', NeuroD 5'-acgcagaaggcaaggtgtc-3', 5'-ctctgcattcatggcttcaa-3', Neurog3 5'-tcatcccttggatgcgctcac-3', 5'-ttacccgcttgggagactgg-3', Pax 4 5'-acctctctgcctgaagacac-3', 5'-tgggcctccaatcagatgatgc-3', Nkx 6.1 5'-tcggacgcccatcttctgg-3', 5'-aggctgccaccgctcgatttg-3', PDX1 5'-gacctttcccgaatggaac-3', 5'-cggttttggaaccagatttt-3' Thermocycling conditions were 30 sec at 94℃, 30 sec at 60℃, and 30 sec at 72℃ for 30~32 cycles preceded by 10 min at 72℃. PCR products were fractionated by 2% agarose gel electrophoresis, stained with loadingstar (Dynebio, Sungnam, Korea), and visualized with

UV light.

Immunocytochemistry

MIN6 were cultured with putrescine and cadaverine in 8-well plates (Lab-Tek, Nalge Nunc International, Naperville, IL, US) and tested with an intracellular immunofluorescent staining flow analysis kit (Imgenex, San Diego, CA, US) according to the manufacturer's protocol. The cells were incubated with specific rabbit anti-MafA antibody (1:100, Santa Cruz Biotechnology) for 2 h at room temperature. After washing the cells were incubated with goat anti-rabbit IgG-FITC (1:500, Invitrogen) for 1 h at 4℃. Cells present in 16 optical fields (200 ×) were examined under a Nikon Eclips TE2000U microscope and analyzed with Nikon NIS Elements Basic Research software. The percentages of positive cells for MafA antigen were obtained and compared to the total number of cells labeled with Hoechst stains.

Statistical analysis

Data were evaluated for statistical significance using SPSS 12.0 for Windows. Values are expressed as the means ± standard deviation (SD). Differences were considered significant when $*P < 0.05$.

RESULTS

Effects of putrescine and cadaverine on insulin secretion of MIN6

We examined the effects of putrescine and cadaverine on the insulin production and secretion of mouse pancreatic

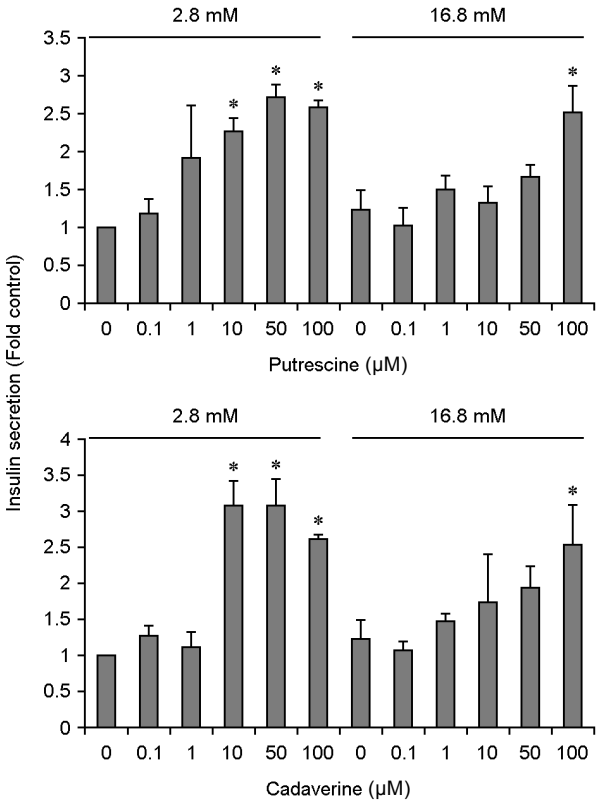

Fig. 1. BIS and GSIS by the MIN6 cells treated with polyamines for 20 min. After equilibration at 2.8 mM glucose, the cells were treated with various concentrations of polyamines in the presence of 2.8 mM (for BIS) or 16.8 mM glucose (for GSIS) for 20 min. The amount of insulin released into culture media was measured by ELISA. Bars represent means ± SD of three independent experiments. *P<0.01 compared with the unstimulated samples.

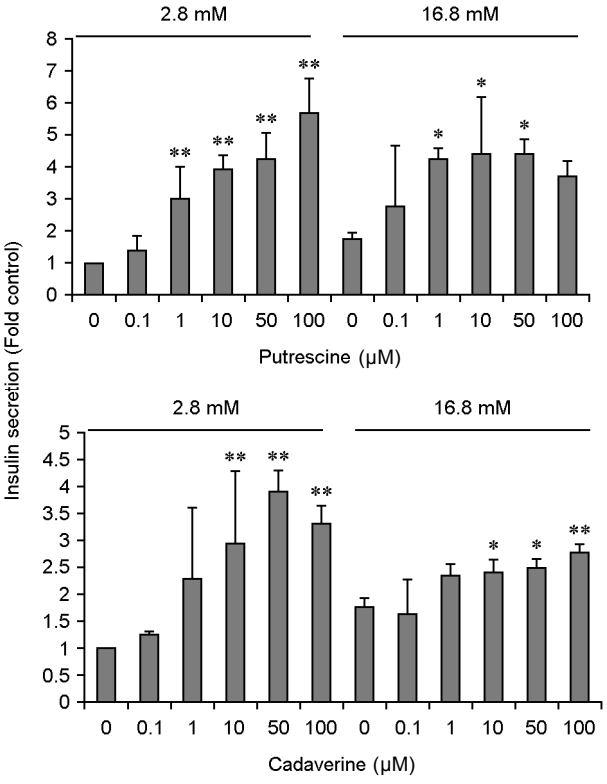

Fig. 2. BIS and GSIS by the MIN6 cells treated with polyamines for 24 h. The cells were pre-treated with various concentration of polyamines in supplemented DMEM for 24 h. After equilibration at 2.8 mM glucose, the cells were cultured in the presence of 2.8 mM (for BIS) or 16.8 mM glucose (for GSIS) for 20 min. The amount of insulin released into culture media was measured by ELISA. Bars represent means ± SD of three independent experiments. *P<0.05 or **P<0.01 compared with the unstimulated sample.

β-cell line, MIN6. To determine the effects of polyamines on the immediate secretion of insulin which had already been produced and stored in intracellular vesicles, we examined the secreted insulin amount after treatment of MIN-6 cells with various concentrations of polyamines for 20 min. Putrescine was found to significantly increase BIS at concentrations above 10 μM (Fig. 1, upper panel). In contrast, at 100 μM concentration of putrescine, GSIS began to increase. Similar to putrescine, cadaverine at concentrations above 10 μM significantly enhanced BIS, yet increased GSIS at only 100 μM concentration (Fig. 1, lower panel). These results indicate that the polyamines stimulate the secretion of insulin-filled vesicles. However, the stimulating effects of these polyamines on BIS were found to be superior to those on GSIS. Next, we examined BIS and GSIS in MIN6 cells 24 h after polyamine treatment to determine whether or not polyamines affect the insulin synthesis and secretion from the cells. The levels of BIS and GSIS in MIN6 cells were found to significantly increase by treatment of 1 μM putrescine, and the levels in the cells treated with 10 μM putrescine were approximately 4 times higher than those in untreated cells (Fig. 2, upper panel). In the case of cadaverine, the levels of MIN6 cell BIS and GSIS began to significantly increase at concentrations above 10 μM (Fig. 2, lower panel). In the case of 24 h treatment, BIS stimulating effects resulting from the polyamines were also found to be greater than those for GSIS. To find out whether or not the enhancement of BIS and GSIS by polyamines is due to the promotion of insulin synthesis at the levels of transcription and/or translation, we examined insulin mRNA and protein expressions. As shown in Fig. 3A, insulin mRNA expression was not affected by the

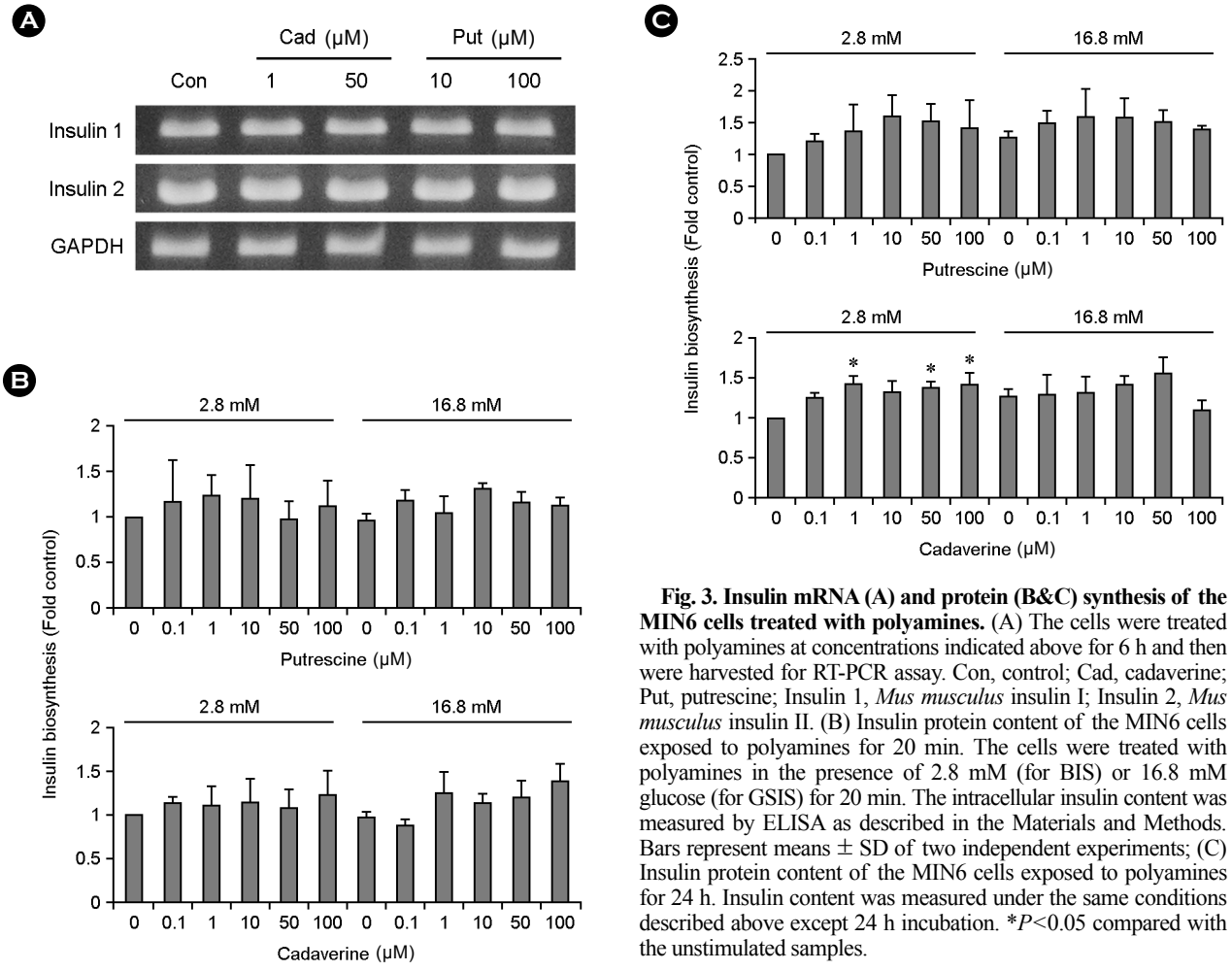

Fig. 3. Insulin mRNA (A) and protein (B&C) synthesis of the MIN6 cells treated with polyamines. (A) The cells were treated with polyamines at concentrations indicated above for 6 h and then were harvested for RT-PCR assay. Con, control; Cad, cadaverine; Put, putrescine; Insulin 1, *Mus musculus* insulin I; Insulin 2, *Mus musculus* insulin II. (B) Insulin protein content of the MIN6 cells exposed to polyamines for 20 min. The cells were treated with polyamines in the presence of 2.8 mM (for BIS) or 16.8 mM glucose (for GSIS) for 20 min. The intracellular insulin content was measured by ELISA as described in the Materials and Methods. Bars represent means ± SD of two independent experiments; (C) Insulin protein content of the MIN6 cells exposed to polyamines for 24 h. Insulin content was measured under the same conditions described above except 24 h incubation. **P*<0.05 compared with the unstimulated samples.

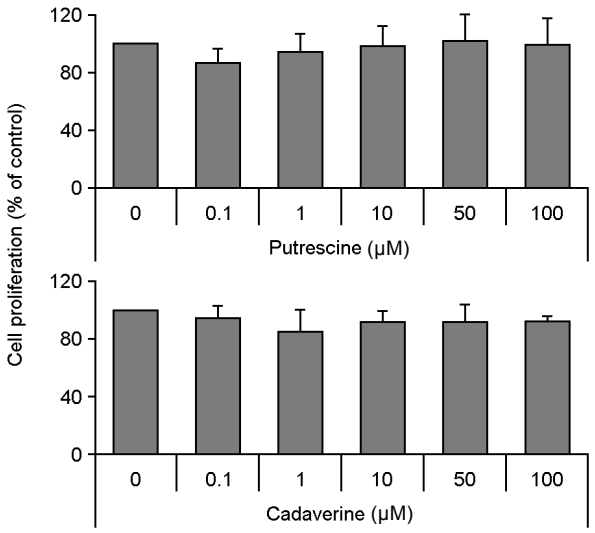

Fig. 4. Cell viability of MIN6 cells treated with polyamines. The cells were treated with various concentrations of polyamines for 24 h and then cell viability was determined by Ez-Cytox assay. Bars represent means ± SD of two independent experiments.

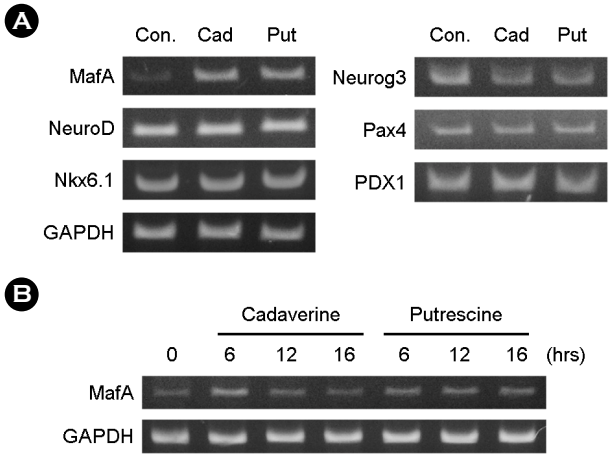

Fig. 5. MafA mRNA expression of the MIN6 cells treated with polyamines. (A) mRNA expression of several transcription factors in MIN6 cells treated with polyamines. The cells were treated with 50 μM cadaverine or 100 μM putrescine for 16 h and then were harvested for RT-PCR assay. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MafA, v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A; Neurog3, neurogenin 3; NeuroD, neurogenic differentiation 1; Pax 4, paired box gene 4; Nkx 6.1, NK6 homeobox 1; PDX1, pancreatic and duodenal homeobox1; (B) MafA mRNA expression of the MIN6 cells treated with polyamines. The cells were treated with 50 μM cadaverine or 100 μM putrescine for 6, 12, 16 h and then were harvested for RT-PCR assay.

treatment of either cadaverine or putrescine to any extent. Similarly, intracellular insulin protein contents in the cells treated with polyamines for 20 min were also unaffected (Fig. 3B). However, basal intracellular insulin contents were slightly increased in the cells treated with 1, 50, or 100 μM cadaverine for 24 h (Fig. 3C). The increased levels were not comparable to those of insulin secretion from the cells treated with cadaverine (Fig. 2). Therefore, it is speculated that exogenous polyamines enhance mainly insulin secretion rather than insulin biosynthesis

Effects of putrescine and cadaverine on MIN6 cell viability and proliferation

Since polyamines are known to promote cell proliferation (Thomas and Thomas, 2003), we examined cell viability and proliferation after treatment with various concentrations (0.1~100 μM) of cadaverine or putrescine to determine whether or not the enhanced insulin secretion may be due to the enhanced cell proliferation. As shown in Fig. 4, the viability and proliferation of MIN6 cells treated with varying concentrations of cadaverine or putrescine for 24 h were not significantly affected. Therefore, the enhanced insulin secretion by the polyamines was not due to enhanced cell proliferation.

Effects of putrescine and cadaverine on the expression of transcription factors involved in insulin synthesis and secretion

Next, we examined the expression of several transcription factors related with insulin synthesis and secretion in order to find the upstream event responsible for the promotion of insulin secretion of MIN6 cells by polyamines. As shown in Fig. 5, among the 6 transcription factors tested, MafA was significantly induced by treatment with cadaverine or putrescine. MafA mRNA expression was significantly induced 6 h after treatment (Fig. 5B). Similarly, MafA protein expression was significantly induced by treatment with polyamines at concentrations above 1 μM (Fig. 6). MafA has been observed to be one of the key transcription factors for insulin secretion (Nishimura et al., 2006).

DISCUSSION

Polyamines are ubiquitous aliphatic cations and exist in pancreatic β-cells at millimolar concentrations (Hougaard

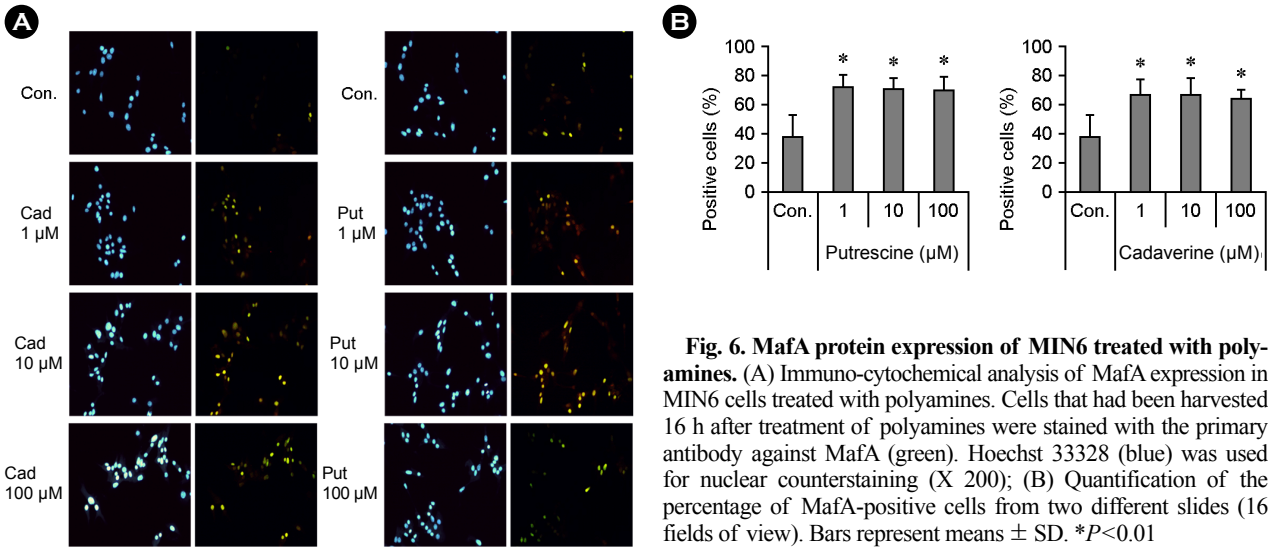

Fig. 6. MafA protein expression of MIN6 treated with polyamines. (A) Immuno-cytochemical analysis of MafA expression in MIN6 cells treated with polyamines. Cells that had been harvested 16 h after treatment of polyamines were stained with the primary antibody against MafA (green). Hoechst 33328 (blue) was used for nuclear counterstaining (X 200); (B) Quantification of the percentage of MafA-positive cells from two different slides (16 fields of view). Bars represent means ± SD. *$P<0.01$

et al., 1986; Welsh and Sjoholm, 1988). In a previous study, the effects of exogenous polyamines on the insulin secretion of islet cells were investigated. Addition of 10 mM spermine suppressed GSIS while addition of spermidine and putrescine had no effect. The authors suggested that inhibition of protein kinase C by spermine is responsible for the suppression of GSIS (Thams et al., 1986). However, they did not examine the effects of polyamines at concentrations below 10 mM because these polyamines exist at millimolar concentrations within the cells: 0.3~0.5 mM for putrescine, 3.9~5.9 mM for spermidine and 1.2~1.9 mM for spermine. Recently, the effects of exogenous addition of 50~250 μM spermidine and spermine on insulin secretion in a mouse β-cell line have been studied and 100 μM spermidine was found to promote insulin secretion, while spermine had no effect (Ohtani et al., 2009).

Based on our findings that exogenous polyamines at micromolar concentrations, which are much lower than the intracellular concentrations, influence insulin secretion, we speculate the possibility of interactions between polyamines and cell surface receptors. Indeed, polyamines such as spermine, spermidine and putrescine have been known to block K^+ channels and thus modulate a resting membrane potential of excitable and non-excitable cells (Williams, 1997). The basic molecular mechanisms for glucose-induced insulin secretion include a closing of ATP-sensitive K^+ channels, an opening of voltage-dependent Ca^{2+} channels and subsequent Ca^{2+} influx, and exocytosis of insulin-filled vesicles. In this regard, it is worthwhile to note that polyamines can interact with K^+ channels. In general, K^+ channel are left active and open in the presence of concentrations of glucose below 5 mM. Therefore, insulin-filled vesicles do not move out of the cell into the extracellular space and thus, BIS does not occur (Brixel et al., 2010). However, our results revealed that the polyamines enhance BIS (Fig. 1). Therefore, it is speculated that cadaverine and putrescine may block K^+ channels. Although it has been reported that cadaverine blocks *E. coli* porins through the similar modulatory actions to those observed in K^+ channel block by polyamines such as spermine and spermidine (Dela Vega and Delcour, 1995), it is unknown whether or not cadaverine interacts with K^+ channels. However, regarding the structural similarity of cadaverine to putrescine, cadaverine may retain the ability to interact with ion channels, just as putrescine does.

In this study, we found that the enhancement of insulin secretion by polyamines may be associated with increased expression of MafA, which has been known to regulate expression of genes involved in insulin production and secretion (Wang et al., 2007). Insulin-positive cells produced *in vitro* (D'Amour et al., 2006; Kroon et al., 2008), and fetal and neonatal beta cells (Hellerstrom and Swenne, 1991; Bliss and Sharp, 1992) are known to be impaired by glucose responsive insulin secretion, indicating their

functional immaturity. MafA is one of the key transcription factors which are involved in such a maturation process (Nishimura et al., 2006). Indeed, significantly enhanced glucose-responsive insulin secretion function was found in MafA overexpressed neonatal rat beta cells, despite the fact that the insulin contents were unchanged (Aguayo-Mazzucato et al., 2011). In another study, in spite of a normal birth, MafA-deficient mice suffered impaired function in secretagogue-stimulated insulin secretion and glucose intolerance (Zhang et al., 2005). In this regard, it is noteworthy that the presence of putrescine and cadaverine increased MafA expression.

The fact that increased BIS levels in cells treated with polyamine for 24 h is much higher than those treated for 20 min (Fig. 1 and 2) suggests the possible involvement of enhanced expression of these transcription factors in the insulin secretion process. We cautiously speculate that, in the case of 20 min-treatment, polyamines may promote BIS through direct interaction with K^+ channels, whereas in the case of 24 h-treatment, polyamines may additionally enhance BIS through up-regulation of transcription factors such as MafA, in addition to the interaction with K^+ channels.

Our results suggest that cadaverine and putrescine enhance the insulin secretion process in MIN6 cells and MafA may be involved in the enhancement. Although MIN-6 cells share many characteristics with freshly isolated pancreatic β-cells (Miyazaki et al. 1990), it is needed to confirm current results by using freshly isolated islets in future study. Furthermore, it is necessary to investigate the underlying mechanisms for the enhancement of insulin secretion by MafA activation and determine whether or not these polyamines interact with ion channels by using *in vitro* patch clamp recordings. This will help us to understand the mechanisms for promotion of insulin secretion by polyamines. Furthermore, future examination of the effects of polyamines on *in vivo* function of pancreatic β-cells can be evaluated for possible use as anti-diabetic compounds.

Acknowledgements

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