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ORIGINAL ARTICLE

Polymyxin B, scavenger of endotoxin, enhances isolation yield and *in vivo* function of islets

Sang G. Park,[1] Jae H. Kim,[2] Ji H. Oh,[3] Han N. Lee,[3] Ho S. Park,[3] Sung S. Chung,[3] Ye J. Lee,[3] Yeon Y. Lee,[3] Hye S. Jung[3] and Kyong S. Park[3]

1 Department of Biomedical Science, CHA University, Yeoksam-dong, Kangnam-gu, Seoul, Korea
2 Division of Endocrinology and Metabolism, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea
3 Department of Internal Medicine, Seoul National University College of medicine, Seoul, Korea

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Correspondence
Kyong S. Park MD, PhD, Department of Internal Medicine, Seoul National University College of Medicine, 28 Yongon-Dong Jongno-Gu, Seoul, 110-744, Korea. Tel.: +82-2-2072-1789; fax: +82-2-3676-8309; e-mail: kspark@snu.ac.kr

Sang Gyu Park PhD, Department of Biomedical Science, CHA University 606-16, Yeoksam-dong, Kangnam-gu, Seoul, 135-081, Korea. Tel.: 82-2-555-5097; e-mail: sgpark@cha.ac.kr

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Summary

Collagenase purified from bacteria has been used to isolate islets for transplantation. However, collagenase is contaminated with small amounts of endotoxin, which induces dysfunction or apoptosis of islets. In this study, we investigated the effects of polymyxin B, endotoxin scavenger, on the yield and quality of isolated islets. It is revealed that polymyxin B neutralized endotoxin *in vitro* and inhibited endotoxin-mediated decreases of the glucose stimulation index. Additionally, adenosine triphosphate (ATP) quantitation, islet regression assay, and caspase-3 activation assay demonstrated that polymyxin B efficiently blocked the toxic effects induced by endotoxin. Thereafter, we isolated mouse islets both with and without polymyxin B and compared total islet equivalents (IEQs), glucose-stimulated insulin release, and ATP content. Polymyxin B enhanced islet recovery, and ATP content of islets, and glucose stimulation index, and reduced TNF-α expression of islets. Marginal transplantation (200 IEQs/mouse) under the kidney capsule of diabetic mice induced normoglycemia in 30% of the polymyxin B group, but not in any mouse of control group. This result suggests that islets isolated with polymyxin B more effectively lower blood glucose levels as compared with control islets. Thus, polymyxin B could serve as a useful agent in the protection of islets from endotoxin-induced inflammation and apoptosis.

Introduction

The application of the Edmonton protocol has pioneered pancreatic islet transplantation as a useful therapeutic strategy for patients suffering from type 1 diabetes mellitus (DM) [1,2]. However, the post-transplantation insulin-independence rate remains low. The low probability of islet engraftment might be resulting from poor islet recovery from donors [3] and early islet losses [4] for instant blood-mediated inflammatory responses (IBMIR) [5,6]. In addition, the collagenase used in islet isolation might damage cellular and extracellular pancreatic components [6,7]. Additionally, exposure to severe environment such as various cytokines or shear stress of transplanted islets might be causing islet dysfunction or low islet engraftments [8–10]. Recently various methods to enhance recovery or engraftment of islets have been tried. This includes the cell-permeable peptide inhibitor of c-Jun N-terminal kinase (JNK), which prevents islet apoptosis after isolation and improves islet grafts [11–13].

Although enzyme quality for islet transplantation has been improved, the collagenase used in islet transplantation procedures may still have mild endotoxin contamination. Endotoxin induces immune-cell activation through toll-like receptor 4 (TLR4) signaling, resulting in the secretion of inflammatory cytokine such as TNF-α

and MCP-1 [14]. Endotoxin contamination is known to be responsible for the failure of human pancreatic islet transplantation because islets damaged by endotoxin undergo apoptosis through activation of mitogen-activated protein kinases (MAPKs) [15–18]. Endotoxin also decreases *in vitro* insulin secretion by glucose stimulation through TLR4 signaling [19]. TLR4 is mainly expressed in ductal epithelium, vascular endothelium, and islet within pancreas, and its expression is up-regulated during islet isolation [20,21]. Suppression or knock-out of TLR4 in islets resulted in decreased inflammatory responses and enhanced allograft survival [21].

The polyamine antibiotic polymyxin B, an inhibitor of protein kinase C (PKC), suppresses endotoxin binding to TLR4 by neutralizing the endotoxin and blocks TNF-α secretion from immune cells [14]. Additionally, polymyxin B has been used as endotoxin scavenger in septic patients [22]. Polymyxin B inhibited PKC activation and nitric oxide (NO) production as reported in previous studies [23,24]. PKC activation mediates TNF-α production by cytokine stimulation and result in enhanced inflammation [25]. Nitric oxide induces islet injury and reduced NO production improves grafted islet function [4,24], Therefore, we hypothesized that ductal injection of polymyxin B could inhibit endotoxin-mediated inflammation of residual immune cells, resulting in improvements in isolation yield and in quality of isolated islets.

In this study, we investigated the effects of polymyxin B on islet recovery, glucose-stimulated insulin release (GSIR), ATP content and cytokine expression of isolated islets. We also evaluated the blood glucose regulating activity of transplanted islets in syngeneic transplantation model.

Materials and methods

Islet isolation and culture

Fifteen-week-old male C57Bl/6 mice, weighing 25–30 g, were purchased from Orient-Bio (Seoul, Korea). Mouse islets were isolated with collagenase digestion followed by Ficoll gradient purification and hand pick-up. Animals were sacrificed by cervical dislocation. Pancreases were exposed and injected with Hanks' balanced salt solution (HBSS; GIBCO BRL, Grand Island, NY, USA) containing 0.5 mg/ml collagenase P (Roche Biochemicals, Basel, Switzerland) in the presence or absence of polymyxin B (10 μm) through the common bile duct until it was distended. Digestion was performed at 37 °C with collagenase P (0.5 mg/ml) for 20 min with gentle agitation. Digestion was terminated by the addition of cold Roswell Park Memorial Institute (RPMI) medium containing 10% FBS and L-glutamine (2 mmol/l, GIBCO BRL). Pancreases were mechanically disrupted by passing tissues through a metal mesh, and islets were purified on Euro-Ficoll (Sigma, St. Louis, MO, USA) gradients by centrifugation at 1200 *g* for 15 min. Islets were hand-picked, counted and scored for size. An algorithm to calculate the 150 μm diameter islet as 1 islet equivalent number (IEQ) was used. Islets were maintained in RPMI 1640 medium supplemented with L-glutamine (2 mM), 10% FBS, 1% penicillin/streptomycin.

TNF-α analysis of isolated islets

Isolated mouse islets (20 IEQ) were immediately stored at −70 °C or maintained in RPMI 1640 medium as described above. For TNF-α analysis, islets were thawed in 1X PBS and sonicated to harvest whole cell extracts. Total cell extracts were centrifuged at 23 000 *g* for 5 min at 4 °C after sonication and supernatants were harvested. For assay of secreted TNF-α, islets were cultured for 24 h and the supernatant was harvested. The concentration of mouse TNF-α was measured with mouse-specific TNF-α ELISA kits (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Results were expressed as mean values of the three independent assays.

In vitro assay of islet function

A total of 10 islets (150 μm) were selected by hand. Glucose-stimulated insulin release was measured and expressed as the stimulation index, which was calculated as the ratio of stimulated (16.7 mM glucose) to basal (1.67 mM glucose) insulin release during 60 min of static incubation in Krebs–Ringer bicarbonate HEPES buffer [26]. Insulin levels were measured with a radioimmunoassay kit (Biosource, Nivelles, Belgium). For analysis of lipopolysaccharide (LPS) effect, 10 islets were incubated for 3 h with LPS (10 ng/ml) in the presence or absence of polymyxin B (10 μM), and GSIR was measured and expressed as the stimulation index as described above. The ATP content of isolated islets was measured with the Bioluminescent Somatic Cell Assay Kit (Sigma Chemicals) following the manufacturer's instructions. ATP quantitation errors resulting from islet mass variability were validated by measuring the DNA concentrations with the PicoGreen dsDNA kit (Molecular Probes, Eugene, OR, USA).

In vivo islet potency

The *in vitro* tests of islet functions were determined immediately before islet transplantation. A single dose of streptozotocin (230 mg/kg; Enzo Life Sciences International Inc, Plymouth meeting, PA, USA) was injected

intraperitoneally to induce diabetes in C57Bl/6 mice (Orient Co. Ltd., Seoul, Korea). Nonfasting blood glucose measurements were taken with a glucose meter (One Touch Ultra Sensor; Lifescan, Milpitas, CA, USA). Mice with sustained hyperglycemia (>350 mg/dl) were used as islet graft recipients, and 200 IEQs were transplanted under the kidney capsule per recipient at 6 to 7 days after streptozotocin injection. Blood glucose levels were measured twice a week from the tail vein. Nephrectomies were performed to determine graft dependence of diabetes reversal. To carry out intraperitoneal glucose tolerance test (IPGTT), the mice were fasted for 12 h. Blood glucose levels were measured at time 0, and immediately thereafter a 20% sterile glucose solution was injected to reach a concentration of 2 g/kg of body weight. Blood was collected at the indicated time points, and glucose levels were measured. Area under the curve (AUC) was estimated by trapezoidal rule.

RT-PCR analysis

Total RNA was isolated using RNeasy kit (Qiagen, Venlo, the Netherlands) from 500 IEQs and was converted to cDNAs using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The resulting cDNAs were used as templates for PCR amplification with specific primer pairs. Total RNA (0.5 μg) was used for cDNA synthesis. Mouse TNF-α and PKC were amplified with their specific primers: TNF-α forward, 5′-TACTGAACTTCGGGGTGATCGGTCC-3′; TNF-α reverse, 5′-ATAGCAAATCGGCTGACGGTGTGGG-3′; PKC forward, 5′-ATGGCTGACGT TTACCCGGCC-3′; PKC reverse, 5′-TCATACTGCACTTTGCAAGATTG-3′; GAPDH forward, 5′-GTGAAGGTCGGTGTGAACGGA-3′;GAPDH reverse, 5′-CCCATCACAA ACATGGG GGCA-3′.

Statistical analysis

Mean values between the control and polymyxin B treated groups were compared with the paired Student's *t*-test. Differences between groups were considered significant when $P < 0.05$.

Results

Effect of polymyxin B on islet toxicity

First, we investigated whether polymyxin B could neutralize the lipopolysaccharide (LPS), endotoxin, *in vitro*. An endotoxin assay using Limulus Amebocyte Lysate (LAL) showed that polymyxin B efficiently neutralized LPS in a dose-dependent manner (1 μM, 58.3 ± 1.21%; 10 μM, 4.82 ± 0.51%; 100 μM, 7.37 ± 0.92% vs. LPS, $n = 5$, $P < 0.001$) (Fig. 1a). As LPS is known to decrease insulin secretion by glucose stimulation *in vitro* through TLR4 signaling, we isolated mouse islets to investigate the effect of LPS and polymyxin B on GSIR [19]. LPS decreased the stimulation index about 25% (3.54 ± 0.32 vs. 4.75 ± 0.43, $n = 6$, $P < 0.05$). Preincubation of LPS with polymyxin B abolished the LPS effects on GSIR (4.48 ± 0.4 vs. 3.54 ± 0.32, $n = 6$, $P < 0.05$). There were no effects of polymyxin B alone on GSIR (4.78 ± 0.48 vs. 4.75 ± 0.43, $n = 6$, not significant) (Fig. 1b). Thereafter, we examined the effect of LPS and polymyxin B on islet toxicity. LPS treatment decreased the ATP content of islets (62.4 ± 1.29 pmol vs. 43.2 ± 0.68 pmol, $n = 5$, $P < 0.05$) (Fig. 1c). Neutralization of LPS by polymyxin B abolished the decreases in ATP content (43.2 ± 0.68 pmol vs. 58.45 ± 3.48 pmol, $n = 5$, $P < 0.05$). Additionally, LPS induced islet regression about twofold compared to controls (31.86 ± 2.22% vs. 16.45 ± 3.46%, $n = 5$, $P < 0.05$) whereas preincubation of LPS with polymyxin B abolished islet regression (31.86 ± 2.22% vs. 19.56 ± 2.16%, $n = 5$, $P < 0.05$) (Fig. 1d). We analyzed caspase-3 activation of islets by LPS. LPS induced maturation of pro-caspase 3 into active caspase 3, whereas preincubation of LPS with polymyxin B inhibited the maturation of caspase 3 (Fig. 1e). As caspase-3 maturation is only induced at apoptosis, this result demonstrated that LPS directly induced islet apoptosis.

Mouse islet isolation using polymyxin B

Because collagenase P for mouse islet isolation is contaminated with endotoxin (approximately 100–1000 EU/mg), we investigated whether polymyxin B could enhance islet isolation yield through neutralization of endotoxin. The infusion of polymyxin B with collagenase P (607.5 ± 37.3 IEQ, head = 25) resulted in higher yield compared to the control group (434.5 ± 21.3 IEQ, head = 25, $P < 0.05$), as seen in Fig. 2a. Islets over 150 μM in size occupy about 80% of total islets. There was no difference in the size distribution of isolated islets between the control and polymyxin B-treated group (Fig. 2b).

In vitro analyses of islets

We further compared insulin release of islets by static glucose stimulation between control and polymyxin B group as described in methods. Ductal injection of polymyxin B with collagenase P enhanced the stimulation index by high glucose of islets about 30% (6.04 ± 0.57 vs. 4.53 ± 0.45, $n = 5$, $P < 0.05$) (Fig. 3a). Interestingly, the ATP content of islets isolated with polymyxin B (88.31 ± 19.4 pmol/20 IEQ, $n = 5$) demonstrated higher levels compared to control islets (60.84 ± 6.3 pmol/20 IEQ, $P < 0.05$), as seen in Fig. 3b. We also compared expression and secretion of TNF-α with ELISA as a representative cytokine of islet

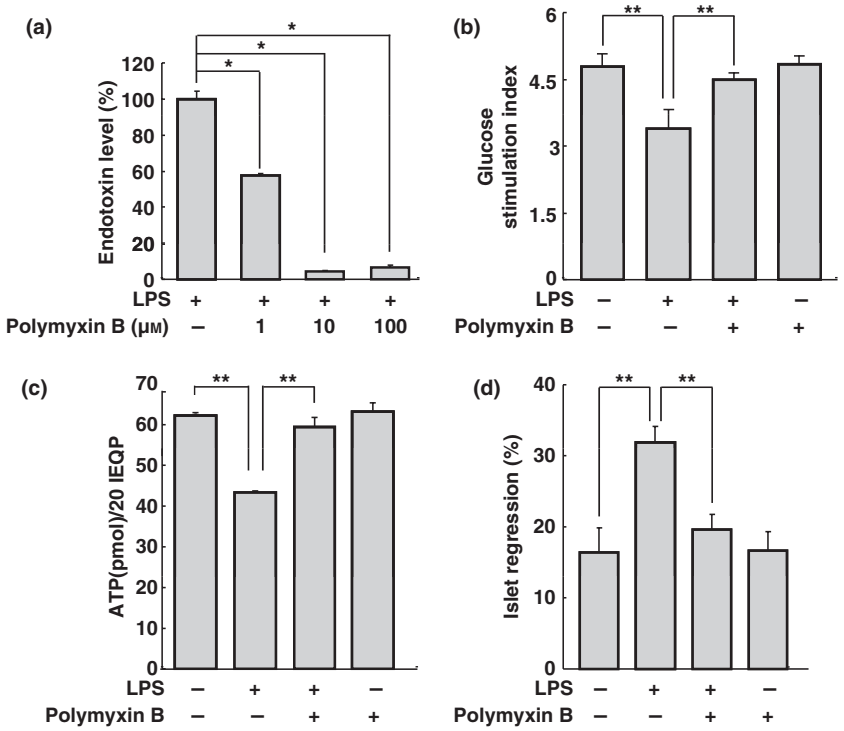

Figure 1 Effect of lipopolysaccharide (LPS) and polymyxin B on islet. LPS (100 ng/ml) was preincubated with either polymyxin B (1, 10, 100 μM) or vehicle for 30 min at room temperature and analyzed (a). LPS (10 ng/ml) was preincubated with either vehicle or polymyxin B (10 μM) for 30 min at room temperature and used to treat mouse islets for GSIR analyses (b) and ATP content (c) for 3 h. The mixture was also used to investigate islet regression and caspase-3 activation for 24 h (d–e). In ATP quantitation assays, islet variability was validated through chromosomal DNA quantification. Islet regression was evaluated by quantification of islet equivalents (IEQs). For caspase-3 activation assay, islets were lysed, and protein extracts (70 μg) were loaded to 14% SDS-PAGE to blot caspase 3 with its specific antibody. Tubulin was used as a loading control. The values are means ± SD values. $*P < 0.01$; $**P < 0.05$.

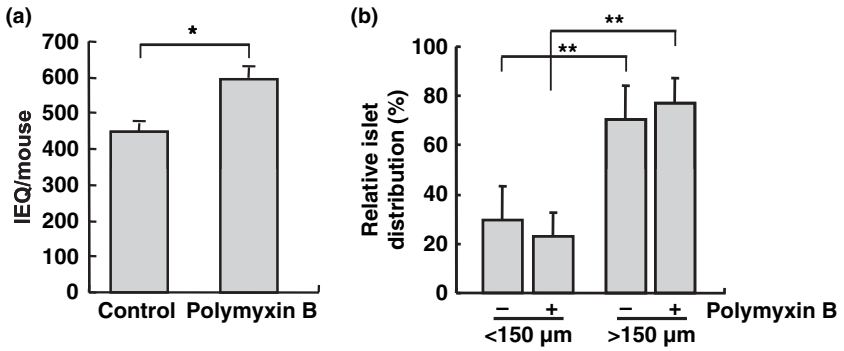

Figure 2 Comparison of islet isolation yield and size distribution. (a) Polymyxin B (10 μM) or vehicle with collagenase P were infused to isolate pancreatic islets of mouse ($n = 25$) as described in methods. (b) Isolated islets were analyzed by size distribution. The values are means ± SD values. $*P < 0.05$;$**P < 0.001$.

inflammation. TNF-α expression levels in polymyxin B-infused islets were markedly decreased compared to control islets (2.04 ± 0.42 pg/μg vs. 5.24 ± 0.82 pg/μg, $n = 5$, $P < 0.001$) (Fig. 3c), and its secretion was also decreased in the polymyxin B group compared to the control group (31.2 ± 2.85 pg/ml vs. 57.3 ± 4.32 pg/ml, $n = 5$, $P < 0.001$) (Fig. 3d). Additionally, we investigated whether mouse TNF-α expression levels were regulated at the transcriptional level. RT-PCR analysis showed that polymyxin B treatment reduced transcript level of TNF-α (Fig. 3e). As it has been known that polymyxin B functions as an inhibitor of mouse PKC, we examined whether polymyxin B could affect mRNA expression of PKC. RT-PCR analysis demonstrated that the concentration of polymyxin B used in this experiment did not have any effect on mRNA expression of PKC (Fig. 3e).

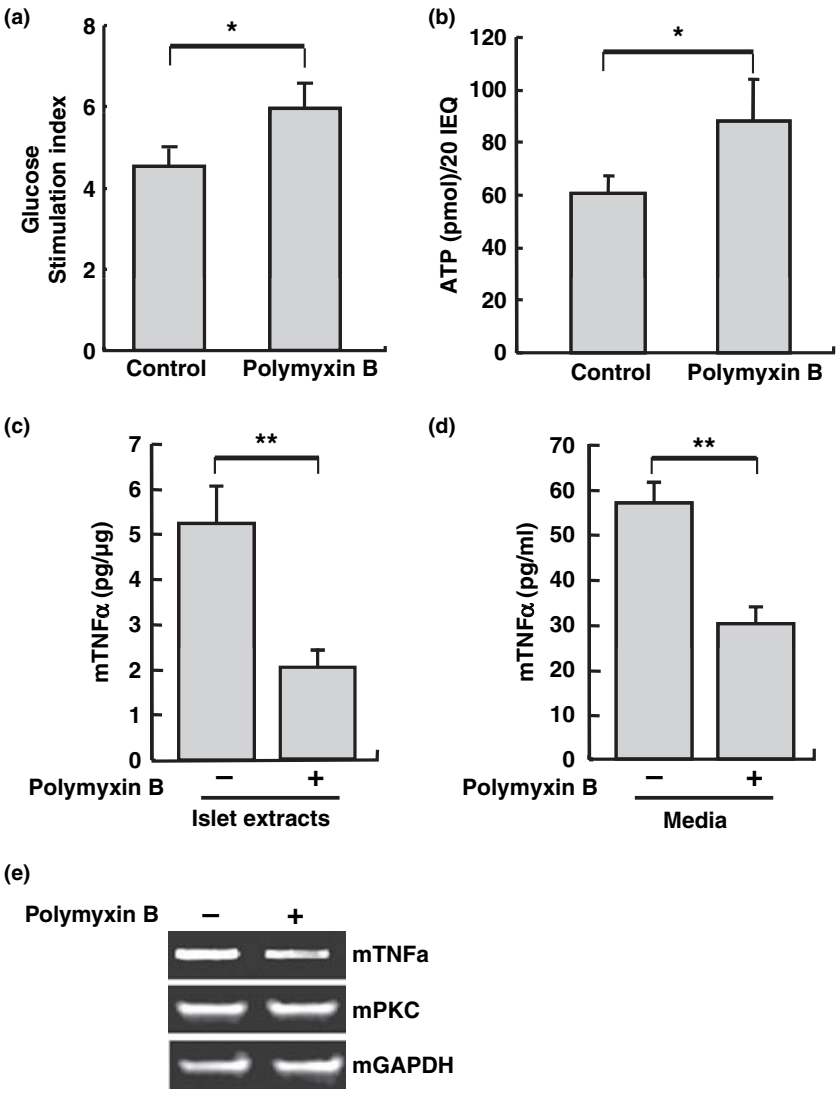

Figure 3 *In vitro* analyses of isolated islets. (a) The stimulation index was calculated as the ratio of stimulated (16.7 mM glucose) to basal (1.67 mM glucose) insulin release ($n = 5$). (b) Total ATP content of 20 handpicked islets (diameter of 100 – 150 μm) was evaluated ($n = 5$). The variability of islet masses in 20 hand-picked islets was validated through chromosomal DNA quantification. (c) 20 islet equivalents (IEQs) were lysed by sonication, and protein extracts were used to quantitate mouse TNF-α expression level using ELISA ($n = 5$). (d) Culture supernatant of islets (1000 IEQs) were harvested, and TNF-α secretion was analyzed using ELISA. (e) Total RNA was isolated with the RNeasy kit, and the transcript levels of mTNF-α and mPKC were analyzed with RT-PCR. GAPDH was used as loading control. The values are means ± SD values. $*P < 0.05$; $**P < 0.001$.

In vivo assays

We transplanted each islet into streptozotocin-induced diabetic C57/Bl6 mice and evaluated their blood glucose regulating activity. Islets isolated with polymyxin B decreased blood glucose levels of mice at 4 weeks although 200 IEQs were marginally transplanted (330 ± 61 mg/dl, $n = 10$), whereas islets isolated with vehicle could not decrease blood glucose levels in transplanted mice (455 ± 61 mg/dl, $n = 10$, $P < 0.05$), as seen in Fig. 4a. Nephrectomies were performed in the polymyxin B group and induced abrupt increases in blood glucose levels (from 344 ± 50 to 480.3 ± 63 mg/dl), whereas nephrectomies in the control group did not result in such increases (from 475 ± 53.5 to 493 ± 55.2 mg/dl) (Fig. 4a). This result suggested that blood glucose regulation of mice in the polymyxin B group was dependent on the transplanted islets. We also analyzed the normoglycemic rate of transplanted mice. Mice transplanted with polymyxin B-treated islets gradually increased up to 30%, while control mice did not exhibit a reversal in diabetes status (Fig. 4b). Additionally, body weights of mice transplanted with islets isolated with polymyxin B gradually increased from week 0 (24.81 ± 0.5 g) to week 5 (26.85 ± 1.13 g, $n = 10$, $P < 0.001$), while control mice did not exhibit significant changes from week 0 (24.71 ± 0.42 g) to week 5 (23.45 ± 1.32 g, $n = 10$) (Fig. 4c). Thereafter, we compared glucose sensitivity between the two groups. At fasting, blood glucose levels of mice transplanted with islets using polymyxin B were significantly lower (276 ± 56 mg/dl, $n = 8$) than blood glucose level of mice transplanted with islets using vehicle (376.2 ± 46.7 mg/dl, $n = 9$, $P < 0.001$) (Fig. 4d). IPGTT revealed that blood glucose

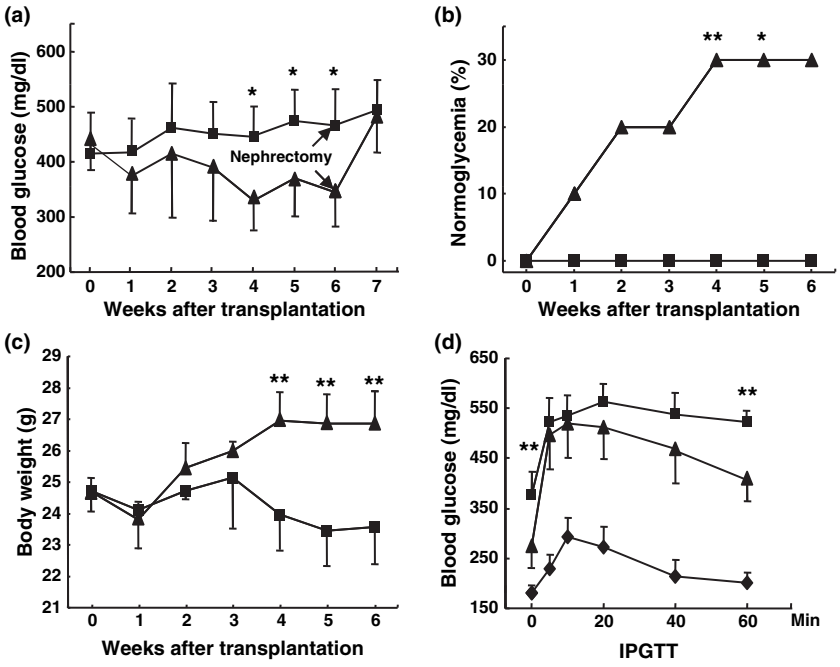

Figure 4 *In vivo* assays of isolated islets. Diabetic mice (C57BL/6) induced with streptozotocin (230 mg/kg) were used to analyze the *in vivo* potency of isolated islets. (a) 200islet equivalents (IEQs) per recipient were transplanted under the kidney capsule. Blood glucose levels were examined twice a week from the tail vein (n = 10–11). Nephrectomies were performed to determine the graft dependence of diabetes reversal. *P < 0.05 versus corresponding value of diabetic control group. (b) The normoglycemic rates of mice transplanted with islets isolated using vehicle or polymyxin B was monitored weekly. *P < 0.05 versus corresponding value of diabetic control group. (c) Body weight change after transplantation was monitored every week. **P < 0.001 versus corresponding value of diabetic control group. (d) The intraperitoneal glucose tolerance test (IPGTT) was performed by injecting 20% sterile glucose solution (2 g/kg) after a 12 h fast at 6 weeks post-transplantation. Blood was collected at the indicated time points, and glucose levels were measured (control, n = 9; polymyxin B, n = 8; normal, n = 5). The diabetic control group was transplanted with vehicle infused islets (filled squares), the diabetic subject group was transplanted with polymyxin B infused islets (filled triangles), and the normal control group did not have transplants (filled diamonds). **P < 0.001 versus corresponding value of diabetic control group. The values are means ± SD values.

levels of mice transplanted with islets isolated with polymyxin B (409.5 ± 25.9 mg/dl, n = 8 at 60 min) were more rapidly removed to peripheral tissues when compared with the control group (521 ± 24.1 mg/dl, n = 9 at 60 min, P < 0.001) (Fig. 4d). The analysis of the AUC clearly showed that blood glucose levels in the polymyxin B group was lower than the control group (20531.4 ± 1271.6 min*mg/dl vs. 26857.9 ± 1538.5 min*mg/dl, n = 8–9, P < 0.05).

Discussion

Islet transplantation is potentially curative in type 1 DM, and it currently requires one or more donors to secure enough islets to transplant into a single recipient. This is despite sufficient number of islets being present in a single pancreas. The necessity for more donors could result from inefficient isolation of pancreatic islets, as current protocols for islet isolation yields at most 3000–6000 IEQs/g. Isolation yield is affected by various factors such as enzyme concentration, digestion time, temperature, status of pancreas, and so on. Recently new methods have been trying to increase isolation yield of islets [27,28]. Also enzyme quality has been improved. Nonetheless, isolation yield of islets from pancreas still needs to be enhanced because collagenase is a little bit contaminated with endotoxin from bacteria. Endotoxin activates residual immune cells at infusion, resulting in secretion of cytokine from immune cells. The NFkB signal of pancreatic beta cells is activated by various cytokines including IL-1beta and IFN-gamma [29]. Activation of NFkB signal by cytokines is known as a key event in the progressive loss of islet cells in type 1 DM [30], and PKC activation is known to mediate cytokine induced TNF-α secretion [25].

Polymyxin B is also known to inhibit endotoxin induced TNF-α secretion [14] and blocks insulin secretion through PKC inactivation [23]. So we tried to find the optimal concentration blocking the binding of endotoxin to immune cells or islets without affecting

insulin secretion of islets. We found that polymyxin B inhibited insulin secretion over 100 μM (data not shown) and could efficiently neutralize 100 ng/ml LPS below 50 μM (Fig. 1a). Accordingly, we investigated whether polymyxin B, an inhibitor of PKC as well as polyamine antibiotic, could enhance isolation yield through islet protection from endotoxin-induced apoptosis and inflammation in this study. Polymyxin B treatment resulted in an increase of isolation yield of about 39%. Furthermore, islets isolated using polymyxin B demonstrated significantly higher GSIR and ATP content as compared with control islets. Higher ATP content was correlated with islet viability and could serve as an index of successful transplantation. We have previously reported that a content of intra-islet ATP correlated with successful transplantation outcomes [31]. We confirmed that high intra-islet ATP content efficiently regulated blood glucose levels in transplanted mice in this study. In addition, we found that polymyxin B inhibited endotoxin-mediated inflammatory responses, leading to decreased TNF-α expression within islets. Usually, islets exposed to endotoxin are typically associated with cytokine production and islet apoptosis [32]. As shown in Fig. 3c and d, islets isolated using polymyxin B demonstrated low expression level and secretion of TNF-α, suggesting that polymyxin B could be applied as a useful agent to inhibit endotoxin-mediated cytokine production through the blockage of inflammatory signals during islet isolation.

Marginal transplantation of islets (200 IEQs) under kidney capsule did not control blood glucose level. However, islets isolated using polymyxin B decreased blood glucose levels in streptozotocin-induced diabetic mice. In addition, nephrectomies of the mice resulted in abrupt increases of blood glucose level (from 344 ± 50 to 480.3 ± 63 mg/dl, $n = 10$, $P < 0.05$), suggesting that decreased blood glucose levels were caused by the transplanted islets, and not attributable to regeneration of endogenous islets. The increases in body weight and efficient glucose tolerance in mice with polymyxin B-transplanted islets suggested that these islets were functionally superior to control islets, resulting in efficient glucose transport from blood to peripheral tissues.

In conclusion, regarding the increased GSIR, ATP content and the decreased inflammatory response of islets additional to increase of isolation yield, polymyxin B could serve as a useful agent to improve islet isolation and transplantation outcomes.

Authorship

SGP: designed research, performed research, wrote paper, collected data, and analyzed data. JHK: designed study, wrote paper, analyzed data. JHO: performed research. HNL: performed research. HSP: performed research. SSC: analyzed data. YJL: performed research. YYL: performed research. HYJ: analyzed data. KSP: wrote paper and analyzed data.

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