Efficacy of DHMEQ, a NF-κB Inhibitor, in Islet Transplantation: I. HMGB1 Suppression by DHMEQ Prevents Early Islet Graft Damage

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Background. Pancreatic islet transplantation (PITx) is an attractive treatment option for restoring appropriate glucose homeostasis in type 1 diabetes patients. Although islet grafts can successfully engraft after PITx, large numbers of islet grafts are required mainly because immune reactions, including inflammation, destroy islet grafts. In these processes, nuclear factor (NF)-κB plays a central role. We hypothesized that the inhibition of NF-κB activation would ameliorate inflammatory responses after PITx and aid successful engraftment.

Methods. To test this hypothesis, a newly developed NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), was used on a syngeneic mouse PITx model. One hundred seventy-five islets from C57BL/6 (B6) mice were transplanted into streptozotocin-induced diabetic B6 mice. The recipient mice were administered DHMEQ for 1, 2, or 3 days after PITx. The underlying mechanisms of DHMEQ on islet graft protection were investigated in an in vitro coculture model of pancreatic islets and macrophages.

Results. With a vehicle treatment, only 11.1% of the islet-recipients achieved normoglycemia after PITx. In sharp contrast, DHMEQ treatment markedly improved the normoglycemic rate, which was associated with the suppression of serum high mobility group complex-1 (HMGB1) and proinflammatory cytokines, including tumor necrosis factor-α, monocyte chemoattractant protein-1, macrophage inflammatory protein-1β, interleukin-1β, and interleukin-6, after PITx. In a murine macrophage-like cell line, DHMEQ inhibited HMGB1-driven activation and proinflammatory cytokine secretion and further prevented death isolated islets after coculture with these activated macrophages.

Conclusions. Inhibition of NF-κB activation by DHMEQ after PITx reduces the HMGB1-triggered proinflammatory responses and results in engraftment of transplanted islets even with fewer islet grafts.

Keywords: DHMEQ, Early graft loss, Nuclear factor-κB, Pancreatic islet transplantation.

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After the introduction of the Edmonton protocol (*1*), pancreatic islet transplantation (PITx) became a feasible treatment option for type 1 diabetes mellitus patients with glycemic instability and refractory hypoglycemia. Approximately 80% of PITx patients are off-insulin at 1 year; however, the protocol requires transplant recipients to receive a sufficient mass of viable islets, which are obtained from two or three donors, to achieve insulin independence. This is chiefly because many islet allografts transplanted into the liver via the portal vein are subsequently destroyed; it has been estimated that 50% to 70% of islets are lost within

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72 hr of the procedure (*2–4*). Hence, an efficient strategy for preventing islet graft loss at or shortly after PITx is of great importance for further improvement of transplant outcome and to enable successful transplantation even when using a reduced islet mass.

When isolated pancreatic islet grafts are transplanted and come into contact with blood, platelet aggregation and activation of coagulation/complement cascades are induced (*5, 6*). These reactions lead to injurious inflammatory and innate immune reactions driven by mediators, such as tissue factor (*7*), monocyte chemoattractant protein (MCP)-1 (*8*), tumor necrosis factor (TNF)-α (*9*), and high mobility group complex-1 (HMGB1) (*10*), subsequently allowing neutrophils and monocytes to infiltrate into the transplanted islets (*11–13*). Macrophages and Kupffer cells, the resident macrophages of the liver, play a key role in these reactions and are activated after PITx (*14, 15*). These activated macrophages secrete proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, TNF-α, and interferon (IFN)-γ, which can directly injure islet grafts (*16*). Within islets, macrophages have been found to produce IL-1 (*17*) and inducible nitric oxide synthase (*18*) in response to TNF-α or IFN-γ. Furthermore, macrophage depletion has been shown to prevent systemic increments of proinflammatory mediators and improved graft survival in a rodent intraportal PITx model (*16*).

Nuclear factor (NF)-κB is a transcription factor that consists of Rel family members, including p65 (Rel-A), p50, p52, c-Rel, and Rel-B. Upon activation, NF-κB homodimers or heterodimers translocate to the nucleus, where the expression of various genes that are crucial for activation, proliferation, or maturation in various cell types is induced (*19, 20*). During inflammation, NF-κB plays a key role in cells, such as macrophages, monocytes, and platelets, which are involved in the early islet graft loss phenomenon (*21*). When triggered by TNF-α, IL-1, lipopolysaccharide, or stimulation provided by various endogenous damage-associated molecules, macrophages are activated via NF-κB, and they release proinflammatory cytokines, such as TNF-α, IL-1, IL-6, or IL-12; the transcription of the relevant genes depends on NF-κB (*22, 23*). The inhibition of NF-κB activation has been demonstrated to prevent proinflammatory cytokine-induced islet apoptosis (*24*).

Here, we tested a hypothesis that inhibition of NF-κB activation shortly after PITx would ameliorate inflammatory responses and thus prevent subsequent loss of islet grafts. To inhibit NF-κB, we used a newly developed low-molecular-weight agent, dehydroxymethylepoxyquinomicin (DHMEQ), which blocks nuclear translocation of NF-κB (*25, 26*).

RESULTS

DHMEQ Ameliorates Normoglycemic Rate in a Marginal Mouse PITx Model

To investigate the effects of NF-κB inhibition by DHMEQ during PITx, we first examined whether DHMEQ ameliorates islet graft loss using a marginal PITx model. After transplantation of 175 syngeneic islets, animals were given DHMEQ intraperitoneally or liposomal-DHMEQ (Lipo-DHMEQ) intravenously at a dose of 20 mg/kg after PITx. Control recipient mice that were administered vehicle (carboxymethylcellulose [CMC] and dimethylsulfoxide [DMSO]) rarely accepted islet grafts (Fig. 1A) and showed a normoglycemic rate (NGR) of 11.1% (Fig. 1B). In contrast, DHMEQ treatment shortly after PITx improved the NGR in a dose-dependent manner. The NGRs were significantly increased when DHMEQ was administered three times (DHMEQ 3×: NGR of 66.7%) or four times (DHMEQ 4×: NGR of 83.3%). A single treatment with Lipo-DHMEQ further enhanced NGR to 85.7%, with six of seven animals accepting the islets grafts (Fig. 1B). No apparent adverse effects were noted in any of the animals during the study.

Transplanted Islet Graft Function After the Marginal Syngeneic PITx

To evaluate the function of transplanted islet grafts, intraperitoneal glucose tolerance test (IPGTT) was performed at 14 days after PITx. In the DHMEQ-treated islet-recipients, blood glucose levels during the IPGTT showed a normal pattern; these were lower than that of the vehicle-treated control islet-recipients at every time point (Fig. 2A). Treatment with DHMEQ significantly improved the function of islet grafts when DHMEQ was administered three or four times or when Lipo-DHMEQ was infused after PITx (Fig. 2B).

DHMEQ Treatment Inhibits Intrahepatic Inflammatory Responses After PITx

To assess inflammatory responses at the transplant site after PITx, liver samples were obtained from islet-recipient animals at 12 hr after PITx, and expression levels of proinflammatory cytokines were assessed by real-time PCR. Up-regulation of TNF-α, MCP-1, macrophage inflammatory protein (MIP)-1β, IL-1β, and IL-6 mRNA expression levels within the liver after PITx were significantly suppressed by DHMEQ treatment compared with those mRNA levels in the control group (Fig. 3).

DHMEQ Suppresses Serum HMGB1 Level

HMGB1, a nuclear protein that is released from damaged tissues or cells (*27, 28*) or is produced by activated macrophages, triggers inflammatory reactions (*29*). We therefore examined serum HMGB1 levels after PITx to examine the role of NF-κB inhibition in PITx. The serum HMGB1 level was significantly elevated at 12 hr after PITx when compared with that of the streptozotocin (STZ)–induced diabetic mice. In contrast, treatment with DHMEQ suppressed HMGB1 elevation in sera ($P<0.05$) after PITx (Fig. 4).

DHMEQ Protects Isolated Pancreatic Islets from Macrophages Activated by HMGB1

Activated macrophages play a crucial role in early graft damage after PITx (*8, 16*). To investigate the protective effects of DHMEQ-mediated inhibition of NF-κB against the damage induced by activated macrophages, we used an in vitro coculture model including pancreatic islets and macrophages. Isolated pancreatic islets were cocultured with HMGB1 prestimulated RAW 264.7 cells with or without DHMEQ treatment. After 24 hr of coculture with non-stimulated RAW 264.7 cells, the number of islets (200 islets) remained unchanged. When islets were cocultured with HMGB1-triggered RAW 264.7 cells (vehicle-treated control), the number of islets reduced to 189±7.3 and 133±15 islets after 12 and 24 hr, respectively. In contrast, addition of DHMEQ to the culture medium significantly prevented

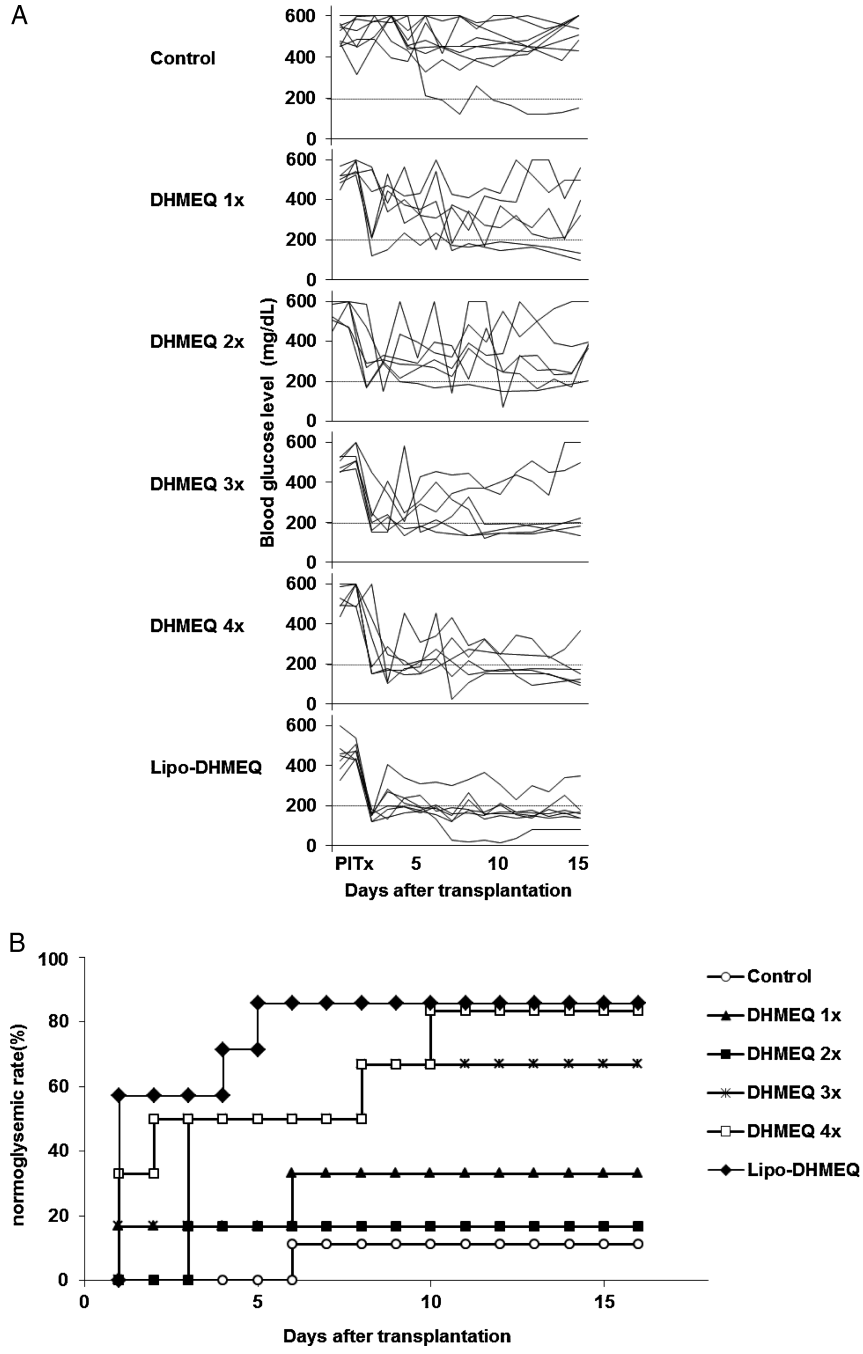

FIGURE 1. DHMEQ ameliorates the NGR in a marginal mouse PITx model. A and B, after 175 pancreatic islets from a B6 mouse were transplanted into the liver of the STZ-induced diabetic B6 mouse, nonfasting blood glucose levels were monitored. Recipient animals were administered DHMEQ (20 mg/kg) intraperitoneally once daily, starting from day 0. DHMEQ 1×: once, on day 0 (n=6; black triangles); DHMEQ 2×: twice, on days 0 and 1 (n=6; black squares); DHMEQ 3×: three times, on days 0, 1, and 2 (n=6; crosses); and DHMEQ 4×: four times, on days 0, 1, 2, and 3 (n=6; white squares). Control vehicle (n=9; white circles) was administered to the control recipient animals. Lipo-DHMEQ was administered intravenously at a dose of 20 mg/kg once immediately after PITx to an additional group of animals (Lipo-DHMEQ: n=7; black diamonds). In this marginal PITx model, vehicle-treated control recipient mice rarely accepted the islet grafts and showed an NGR of 11.1%. In contrast, DHMEQ treatment shortly after PITx showed improved NGR in a dose-dependent manner and significantly improved the NGR of the DHMEQ 3× (NGR: 66.7%) and DHMEQ 4× (NGR: 83.3%) groups compared with control. Treatment with Lipo-DHMEQ further improved the NGR (85.7%), with six of seven animals accepting the islet grafts despite a single dose of DHMEQ (*P<0.05 vs. vehicle-treated control group; Kaplan–Meier log-rank test).

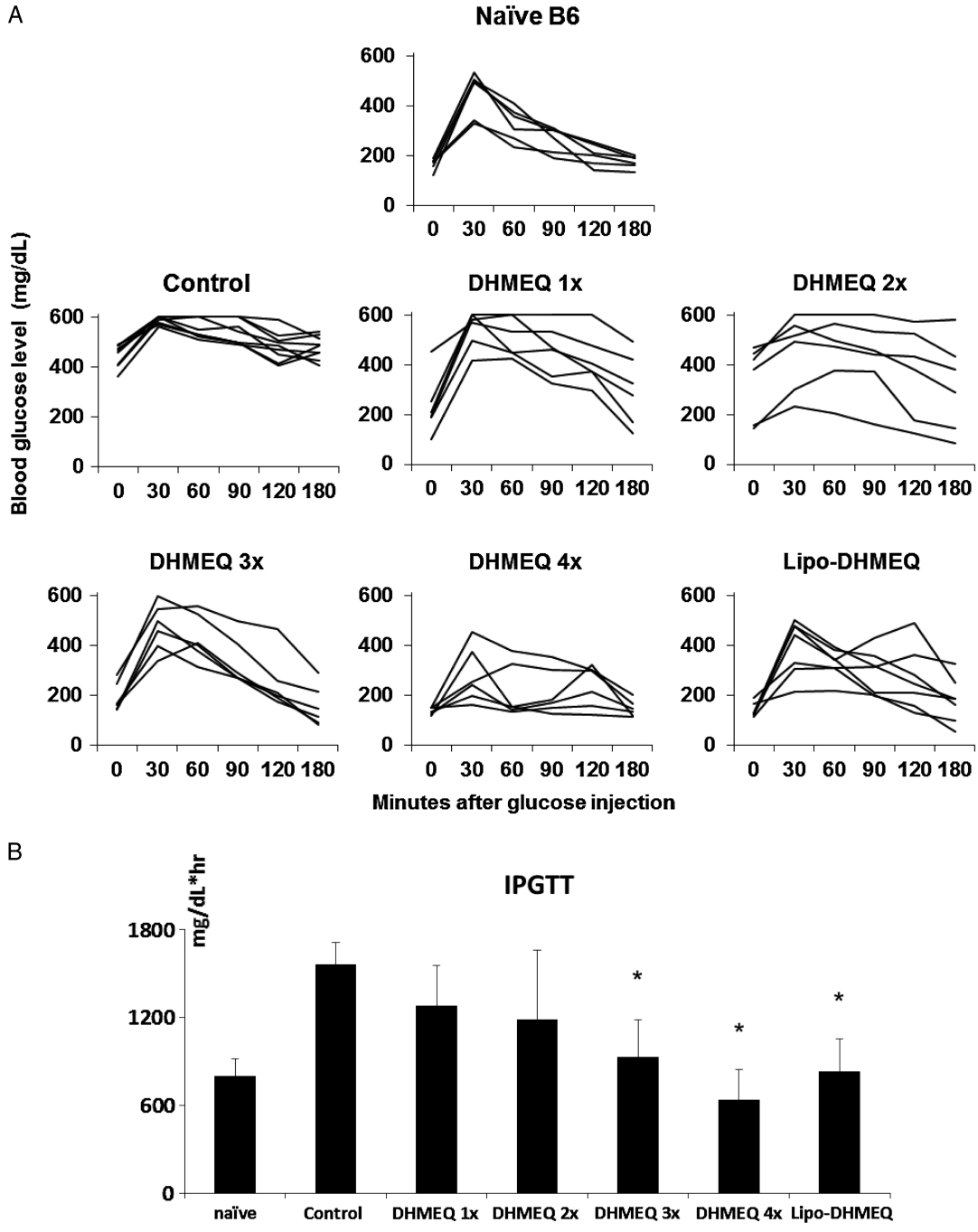

FIGURE 2. Function of transplanted islets after marginal syngeneic PITx. A, an IPGTT was performed at 14 days after PITx. Blood glucose levels during the IPGTT showed a normal pattern in DHMEQ-treated recipients, which had significantly lower blood glucose levels than vehicle-treated controls at every time-point. B, treatment with DHMEQ significantly improved the function of islet grafts in the DHMEQ 3×, DHMEQ 4×, and Lipo-DHMEQ groups compared with the control (*P<0.05 vs. vehicle-treated control group).

islet loss when examined at 24 hr after coculture (194±2.8 islets; P<0.05) compared with the vehicle-treated control group (Fig. 5A). Furthermore, addition of DHMEQ significantly suppressed the protein levels of HMGB1, IL-6, and TNF-α in the coculture supernatants compared with those of the vehicle-treated control group (Fig. 5B–D).

DISCUSSION

In this study, we used DHMEQ, a newly developed low-molecular-weight agent derived from the weak antibiotic epoxyquinomicin (*25*), as an NF-κB inhibitor. DHMEQ inhibits NF-κB activation at the nuclear translocation level, but this process does not involve the degradation of IκB or

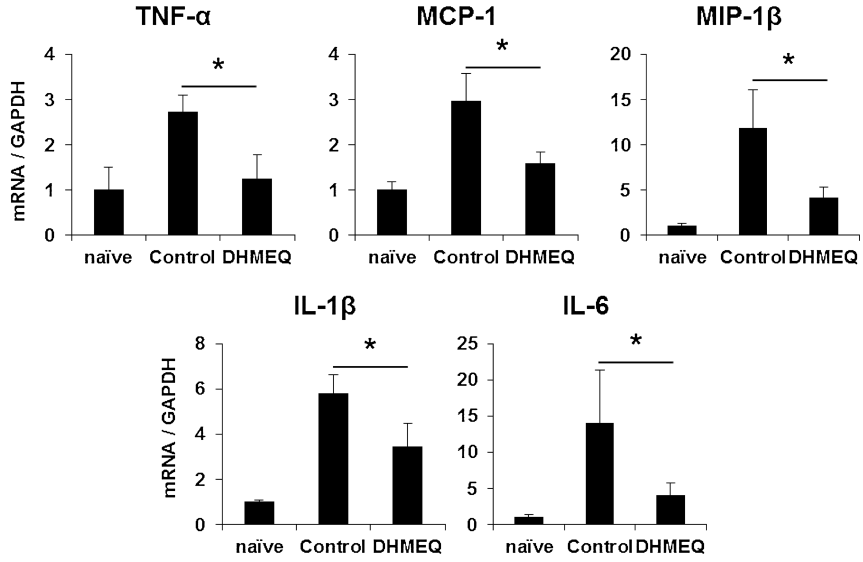

FIGURE 3. Treatment with DHMEQ inhibits intrahepatic inflammatory responses. Intrahepatic inflammatory responses were assessed by real-time PCR. Liver samples were obtained from recipient animals treated with DHMEQ intraperitoneally at 12 hr after PITx and from nondiabetic healthy mice. mRNA expression of TNF-α, MCP-1, MIP-1β, IL-1β, and IL-6 were suppressed by treatment with DHMEQ compared with GAPDH mRNA expression (*P<0.05 vs. control, n=5 respectively).

a c-Jun N-terminal kinase– or caspase-activating pathway (*26*). Furthermore, Yamamoto et al. (*30*) found that DHMEQ directly binds to components of the Rel family, such as p65 (Cys38 residue), cRel, RelB, and p50, at a residue that is essential for DNA binding. The specific and direct binding of DHMEQ to these proteins may explain its highly selective NF-κB inhibition and its low toxicity. With regard to the anti-inflammatory effects of DHMEQ, we have previously demonstrated that treatment of animals with 20 mg/kg DHMEQ resulted in suppression of TNF-α and IL-6 production and a marked improvement of survival rate after intestinal ischemia-reperfusion injury in rats (*31*). The anti-inflammatory effects of DHMEQ were also demonstrated in human synovial cells (*32*) and keratinocytes (*33*) and in murine models of arthritis (*34*), uveoretinitis (*35*), and colitis (*36*). Furthermore, it has been shown that, in INS-1 cells, a β-cell line, DHMEQ prevents cell dysfunction induced by TNF-α (*37*). Here, we have demonstrated that a short course of DHMEQ immediately after PITx improves NGR in a dose-dependent manner when using a mouse marginal PITx model in which only 175 islets were transplanted. This effect was associated with significantly reduced mRNA expression levels of proinflammatory cytokines, which are known to induce islet cell damage, in the islet-recipient's liver (*28, 38*).

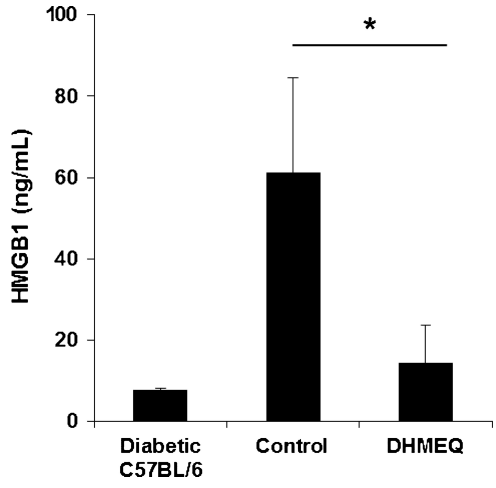

FIGURE 4. Treatment with DHMEQ suppresses the serum HMGB1 level after PITx. Blood samples were collected via the hearts of recipient animals at 12 hr after marginal PITx, and the serum HMGB1 level was measured by ELISA. Blood samples from STZ-induced diabetic B6 mice were similarly collected at 6 days after STZ injection. In the vehicle-treated control animals, serum HMGB1 levels were elevated. In contrast, treatment with DHMEQ significantly suppressed serum HMGB1 levels compared with both control and diabetic nontransplanted B6 control animals (*P<0.05 vs. control, n=5 in all groups).

In addition to up-regulation of proinflammatory cytokines, we also found that the serum HMGB1 level was elevated shortly after PITx in the islet-recipient mice. This finding was similar to that of Matsuoka et al. (*10*), who found that HMGB1 was released into the circulation soon after PITx and that the administration of HMGB1-specific antibody prevented early islet graft damage. Moreover, Gao et al. (*39*) demonstrated that treatment with an anti-HMGB1 monoclonal antibody reduced TNF-α and IL-1β production and improved islet viability. To elucidate the methods underlying the DHMEQ-mediated protective effect after PITx, we focused on the association between HMGB1 and macrophage activation. HMGB1, a nuclear protein, is secreted by activated immune cells, such as macrophages, dendritic cells, and natural killer cells, in response to injury or inflammation (*27, 40*). Binding of the

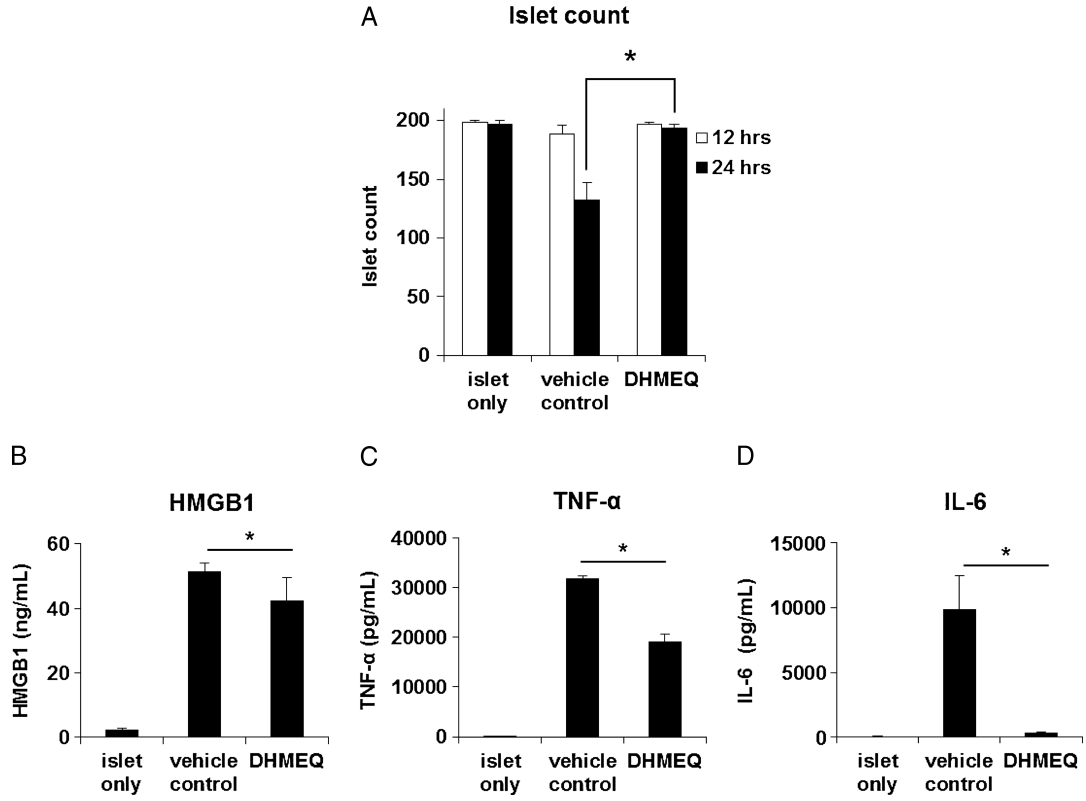

FIGURE 5. NF-κB inhibition by DHMEQ protects isolated pancreatic islets. A, RAW 264.7 cells were cultured in a 96-well plate and pretreated with HMGB1 (20 μg/mL) for 2 hr. The HMGB1-treated RAW 264.7 cells were washed with phosphate-buffered saline solution and were seeded along with 200 pancreatic islets isolated from B6 mice. Cells were then incubated with or without DHMEQ (5.0 μg/mL) at 37°C, in 5% CO_2 and a humidified atmosphere; the supernatants from each dish were then harvested and assessed after 24 hr. After coculture with nonstimulated RAW 264.7 cells, the number of islets was not reduced. In the vehicle (0.5% CMC)–treated control group, the number of islets was reduced after coculture to 189±7.3 islets (mean±SD) after 12 hr and 133±15 islets after 24 hr. In contrast, addition of DHMEQ to the coculture medium significantly suppressed the islet loss during the 24 hr coculture compared with those of the vehicle-treated control group (*P<0.05 vs. vehicle-treated control group). B, protein levels of HMGB1, TNF-α, and IL-6 in the coculture supernatants were evaluated by ELISA. Treatment with DHMEQ significantly suppressed the production of HMGB1, TNF-α, and IL-6 compared with those of the vehicle-treated control group (*P<0.05 vs. vehicle-treated control group).

secreted HMGB1 to the Toll-like receptors 2 and 4 or the receptor for advanced glycation end-products induces NF-κB activating signaling (*29, 41*), which elicits activation of immune cells and promotes subsequent inflammatory responses (*28, 42*). HMGB1 is not only actively secreted by macrophages and other immune cells but also is passively released from damaged tissues or cells, including β-cells (*28*). Pancreatic islets contain high levels of HMGB1, at about 20 times the level of that in other organs, such as the thymus, lung, spleen, and liver (*10*). Thus, the observed elevation of serum HMGB1 after PITx could be due to the activation of immune cells and/or HMGB1 release from the damaged islet grafts.

In the present study, we have demonstrated that treatment with DHMEQ significantly suppressed serum HMGB1 levels shortly after PITx and improved the NGR in a mouse syngeneic PITx model. Based on these findings, we then investigated the association between released HMGB1 and macrophage-induced islet cell damage. First, we examined whether DHMEQ inhibits activation of macrophages upon stimulation with HMGB1 (20 μg/mL). Addition of DHMEQ to the culture media at a dose range of 2.5 to 10 μg/mL significantly inhibited HMGB1-induced TNF-α secretion in RAW 264.7 cells (data not shown). This result was in line with the findings shown by Suzuki et al. (*43, 44*), who found that DHMEQ inhibited secretion of proinflammatory cytokines, inducible nitric oxide synthase expression, and phagocytosis after lipopolysaccharide stimulation in RAW 264.7 cells and mouse bone marrow–derived macrophages. It has been shown that the proinflammatory cytokine, IL-1, secreted by activated macrophages, induces pancreatic β-cell necrotic death and HMGB1 release (*28*). Moreover, TNF-α and IFN-γ prime macrophages to secrete proinflammatory cytokines to increase their killing ability (*45, 46*). To elucidate the protective effect of DHMEQ against macrophage-induced islet injury, we set up a coculture model of HMGB1-activated RAW 264.7 macrophages and isolated pancreatic islets. We showed that DHMEQ prevented islet death in this in vitro model and that the effect was associated with a significant suppression of HMGB1, IL-6, and TNF-α (Fig. 5).

To clarify whether the effect was due to the direct protection of the cultured isolated pancreatic islets by

DHMEQ, we cultured islets in the presence of IFN-γ, TNF-α, and IL-1β (20 ng/mL of each) with or without DHMEQ. After stimulation with these proinflammatory cytokines, the cultured pancreatic islets died concomitantly with the increase in HMGB1 concentration in the culture supernatant. However, the addition of DHMEQ to the culture media did not prevent islet death or HMGB1 elevation (data not shown). Taken together, these findings indicate that DHMEQ treatment inhibited HMGB1-induced macrophage activation and subsequently protected islets from the damage caused by proinflammatory cytokines released by activated macrophages. Thus, it seems likely that, after transplantation of pancreatic islets into the liver, HMGB1 is released from activated macrophages and also from damaged or destructed islet grafts. HMGB1 activates macrophages, which then exacerbates early graft loss.

In this study, an intravenous formulation of Lipo-DHMEQ was tested; here, we have demonstrated that recipient animals treated with Lipo-DHMEQ became normoglycemic at earlier time points after PITx. Although the function of islet grafts was well maintained when naked DHMEQ was administered four times, there was no significant differences between the Lipo-DHMEQ and DHMEQ 4× groups as assessed by NGR (P=0.915) and IPGTT blood glucose levels (P=0.142). However, total dosages of DHMEQ administered in these two groups were different (20 mg/kg for Lipo-DHMEQ vs. 80 mg/kg for DHMEQ 4×); therefore, the Lipo-DHMEQ formula appears to have a stronger effect than the naked DHMEQ. Liposomes biologically target mononuclear phagocytic cells, such as macrophages (*47, 48*). Given the pivotal role of macrophages in inflammation (*48*), the pharmacologic characteristics of liposomes may have caused the difference in the potency of the different DHMEQ formulations. Understanding the pharmacokinetics of different formulations of DHMEQ, with different administration routes, is important for clinical application of DHMEQ; such studies are currently under progress.

The results of our study indicate that control of macrophage activation by NF-κB inhibition can protect transplanted islet grafts from damage caused by intraportal PITx. This is essential for shutting down the undesirable negative feedback loop to facilitate successful PITx with fewer islet grafts. We conclude that inhibition of the initial activities of NF-κB by a novel NF-κB inhibitor, DHMEQ, may facilitate development of a treatment strategy that enables successful PITx for multiple diabetic recipients, even from a single donor.

MATERIALS AND METHODS

Animals

All experiments were approved by the Institutional Animal Care Committee, and the study was conducted according to the Guidelines for the Care and Use of Laboratory Animals of Hokkaido University. Male C57BL/6 (B6: H-2^b) mice were purchased from SLC (Shizuoka, Japan). The mice were maintained in a specific pathogen-free facility and were used for experiments at 12 to 15 weeks of age.

Reagents

DHMEQ was synthesized as described previously (*49*). DHMEQ was dissolved in DMSO (Sigma-Aldrich, St. Louis, MO) and stored at –80°C until use. DHMEQ stock solution was dissolved in 0.5% CMC (Sigma-Aldrich) solution. Lipo-DHMEQ was prepared for use as follows. Cholesterol, 1,2-distearoyl-*sn*-glycero-3-phosphocholine, and *N*-(carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-*sn*-glycero-3-phospho-ethanolamine (NOF, Tokyo, Japan) were dissolved at a 3:6:1 molar ratio in ethanol. After DHMEQ addition, the mixtures were completely dissolved at 65°C. The solvent was removed by evaporation, resulting in a lipid film, and the film was then hydrated by adding saline solution for 20 min at room temperature. The lipid dispersion was sonicated to produce liposomes. To remove unincorporated DHMEQ, the liposomal suspension was purified by centrifugation. Purified HMGB1 was purchased from Shino-Test (Kanagawa, Japan). Recombinant mouse IFN-γ, TNF-α, and IL-1β were purchased from the Sigma-Aldrich.

Pancreatic Islet Isolation

The pancreatic islets of B6 mice were isolated as described previously (*24*). Purified islets that were 150 to 200 μm in diameter were used further. Pancreatic islets for PITx were cultured overnight in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Sigma-Aldrich) at 37°C, with 5% CO_2 and in a humidified atmosphere.

Cell Preparation, Stimulation, and Coculture

The murine macrophage-like cell line RAW 264.7 was obtained from the American Type Culture Collection (Manassas, VA). These cells were cultured in Dulbecco's Modified Eagle Medium (Sigma-Aldrich) supplemented with 2% albumin from bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Sigma-Aldrich). Cells were then seeded in each well of a 96-well plate and pretreated with HMGB1 (20 μg/mL) for 2 hr. After this treatment, the cells were washed with phosphate-buffered saline solution three times and were added to a 35-mm cell culture dish (IWAKI, Tokyo, Japan) at a density of 2×10^6 cells/mL, along with 200 pancreatic islets isolated from B6 mice, in a final volume of 1.5 mL. The culture dishes were incubated at 37°C, with 5% CO_2 and at humidified atmosphere. Supernatant fluids from each dish were harvested and used for analysis.

Diabetes Mellitus Induction

B6 islet-recipient mice were rendered diabetic by intraperitoneal administration of 180 mg/kg STZ (Sigma-Aldrich) 5 to 7 days before PITx. Blood samples were collected via the tail vein, and the blood glucose levels were monitored with the Accu-Chek blood glucose monitor (Roche Diagnostics K.K., Tokyo, Japan). Diabetes was considered to be established when the blood glucose level of two consecutive measurements exceeded 450 mg/dL.

PITx and Treatment Protocol

The isolated islets were washed with RPMI 1640 medium containing 10% fetal calf serum and were counted and transplanted into the recipient liver via the portal vein.

The recipient animals were given DHMEQ (20 mg/kg) intraperitoneally once daily, starting from day 0 as follows. The DHMEQ 1× group received DHMEQ once, on day 0; DHMEQ 2×: twice, on days 0 and 1; DHMEQ 3×: three times, on days 0, 1, and 2; and DHMEQ 4×: four times, on days 0, 1, 2, and 3. Control vehicle (CMC and DMSO) was administered four times on days 0, 1, 2, and 3 in the control group. An additional group of mice received Lipo-DHMEQ intravenously at a dose of 20 mg/kg, once, immediately after PITx, on day 0. Normoglycemia was defined when the blood glucose level in these mice registered at below 200 mg/dL for 2 consecutive days.

Intraperitoneal Glucose Tolerance Test

IPGTT was performed in the islet-recipient animals at 14 days after PITx. Animals were given glucose solution intraperitoneally at a dose of 2 g/kg after 6 hr of fasting. Blood glucose levels were monitored immediately before and again at 30, 60, 90, 120, and 180 min after the glucose injection. Naïve B6 mice were used as controls for this procedure.

Measurement of Proinflammatory Cytokines and HMGB1

IL-1β, IL-6, and TNF-α protein levels in the culture supernatant were quantified by enzyme-linked immunosorbent assay (ELISA) using OptEIA Sets (Becton Dickinson, Franklin Lakes, NJ). All measurements were performed in duplicate. The HMGB1 levels in the serum of animals or

supernatant of cell culture medium were also measured by ELISA as described previously (*50*).

Real-time PCR

Liver samples from the recipient animals treated with DHMEQ intraperitoneally were obtained at 12 hr after PITx, snap frozen in liquid nitrogen, and stored at -80°C until use. Tissue samples were subjected to total RNA extraction using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized from 1 μg total RNA using the Omniscript RT Kit (Qiagen, Valencia, CA) with an oligo(dT)-20 primer (TOYOBO, Osaka, Japan). For relative quantification by PCR, each cDNA product was analyzed in a LightCycler (version 1.4) using a QuantiTect SYBR Green PCR Kit (Qiagen) along with 0.5 μM of specific primers (Invitrogen) in a 20 μL reaction volume. PCR parameters were as follows: 94°C for 15 min followed by 40 cycles of 94°C for 15 s, 60°C for 20 s, and 72°C for 30 s. The relative amounts of the target gene mRNAs (viz., IL-1β, IL-6, TNF-α, MIP-1β, and MCP-1) were normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and to the corresponding mRNA level in naïve livers. The following primers were used: mouse TNF-α forward 5′-ACCCTCACACTCAGATCATC-3′ and reverse 5′-GAGTAGACAAGGTACAACCC-3′, mouse IL-1β forward 5′-GGATGAGGACATGAGCACCT-3′ and reverse 5′-AGCTCATATGGGTCCGACAG-3′, mouse MCP-1 forward 5′-TCCCAATGAGTAGGCTGGAG-3′ and reverse; 5′-TCTGGACCCATTCCTTCTTG-3′, mouse IL-6 forward 5′-CAAAGCCAGAGTCCTTCAGAG-3′ and reverse 5′-GCCACTCCTTCTGTGACTCC-3′, mouse MIP-1β forward 5′-CCCACTTCCTGCTGTTTCTC-3′ and reverse 5′-GTCTGCCTCTTTTGGTCAGG-3′, and mouse GAPDH forward 5′-TACACTGAGGACCAGGTTGT-3′ and reverse 5′-CTGTAGCCGTATTCATTGTC-3′. The relative fold-increase was calculated using the standard curve method.

Statistical Analysis

Differences in the NGR between groups were evaluated by log-rank test using a Kaplan–Meier method. Quantitative results are presented as mean±SD. Statistical analyses were carried out using a Student's *t* test. $P<0.05$ was considered statistically significant.

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