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ORIGINAL RESEARCH

Innate Immune System Regulated by Stimulator of Interferon Genes, a Cytosolic DNA Sensor, Regulates Endothelial Function

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BACKGROUND: Sterile inflammation caused by metabolic disorders impairs endothelial function; however, the underlying mechanism by which hyperglycemia induces inflammation remains obscure. Recent studies have suggested that stimulator of interferon genes (STING), a key cytosolic DNA sensor in the innate immune system, contributes to the pathogenesis of inflammatory diseases. This study examines the role of the STING in endothelial dysfunction in streptozotocin-induced diabetic mice.

METHODS AND RESULTS: Injection of streptozotocin promoted the expression of STING and DNA damage markers in the aorta of wild-type mice. Streptozotocin elevated blood glucose and lipid levels in both wild-type and STING-deficient mice, which showed no statistical differences. Genetic deletion of STING ameliorated endothelial dysfunction as determined by the vascular relaxation in response to acetylcholine ($P<0.001$) and increased endothelial nitric oxide synthase phosphorylation in the aorta ($P<0.05$) in STZ-injected mice. Endothelium-independent vascular response to sodium nitroprusside did not differ. Treatment with a direct STING agonist, cyclic GMP-AMP, or mitochondrial DNA increased inflammatory molecule expression (eg, *VCAM1* and *IFNB*) and decreased endothelial nitric oxide synthase phosphorylation in human umbilical vein endothelial cells, partially through the STING pathway. Cyclic GMP-AMP significantly impaired endothelial function of aortic segments obtained from wild-type mice, which was ameliorated in the presence of C-176, a STING inhibitor, or a neutralizing interferon-β antibody. Furthermore, the administration of C-176 ameliorated endothelial dysfunction in STZ-induced diabetic mice ($P<0.01$).

CONCLUSIONS: The DNA damage response regulated by STING impairs endothelial function. STING signaling may be a potential therapeutic target of endothelial dysfunction caused by hyperglycemia.

Key Words: diabetes ■ endothelial function ■ inflammation ■ STING

Due to lifestyle changes, the prevalence of metabolic diseases has increased worldwide. Despite advances in therapeutics, cardiovascular complications pose a severe risk in these patients. The impairment of endothelial function stimulates the development of cardiovascular complications associated with atherosclerosis.[1] Therefore, the prevention of endothelial dysfunction caused by metabolic disorders is the most important therapeutic strategy. Recent studies have suggested that chronic inflammation

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CLINICAL PERSPECTIVE

What Is New?

- The expression of stimulator of interferon genes, which plays important roles in the DNA-mediated innate immune responses, increases in the aorta of streptozotocin-induced diabetic mice.
- Activation of stimulator of interferon genes promotes inflammatory molecule expression in endothelial cells and impairs endothelium-dependent vascular relaxation, leading to the development of endothelial dysfunction.
- Genetic deletion or pharmacologic blockade of stimulator of interferon genes ameliorated endothelial dysfunction in streptozotocin-induced diabetic mice.

What Are the Clinical Implications?

- DNA damage response, which is regulated by the stimulator of interferon genes signaling pathway, may explain the link between innate immunity and chronic inflammation in the vascular system caused by hyperglycemia and may be a potential therapeutic target for endothelial dysfunction.

Nonstandard Abbreviations and Acronyms

cGAMP	cyclic GMP-AMP
EC	endothelial cell
eNOS	endothelial nitric oxide synthase
HUVEC	human umbilical vein endothelial cell
IRF3	interferon regulatory factor 3
mtDNA	mitochondrial DNA
STING	stimulator of interferon genes
TBK1	TANK-binding kinase 1

caused by metabolic diseases such as diabetes promotes endothelial dysfunction[2–4]; however, the mechanism by which metabolic diseases cause inflammation are not fully understood.

Accumulating evidence suggests that DNA damage and the release of endogenous DNA fragments under excessive nutrient levels, such as those in hyperglycemia and hyperlipidemia, provoke sterile inflammation,[5–11] which involves several pattern recognition receptors functioning as self-defense systems.[12] Besides immune cells, such as macrophages, endothelial cells (ECs) also express pattern recognition receptors as part of the immune system.[13] ECs line blood vessels and act as part of the first line of defense against circulating pathogens and self-derived elements.

Stimulator of interferon genes (STING) plays a pivotal role in the innate immune system. In the innate immune response, STING recognizes cyclic GMP-AMP (cGAMP), which is generated by the interaction of cGAMP synthase and DNA fragments in the cytosol, and activates the interferon regulatory factor 3 (IRF3) and nuclear factor-κB pathways.[14,15] Consequently, STING triggers the production of type I interferons and cytokines, which are among the most important self-defense molecules.[16] Additionally, recent studies have demonstrated that STING contributes to the pathogenesis of various inflammatory diseases by recognizing endogenous DNA fragments released during aging, overnutrition, and others.[17–20] However, the role of STING in the development of EC dysfunction has not yet been fully examined.

In this study, we hypothesized that STING contributes to the development of endothelial dysfunction. Accordingly, we examined vascular responses in streptozotocin-injected wild-type and STING-deficient (*Sting*$^{-/-}$) mice and performed several in vitro studies using human umbilical vein endothelial cells (HUVECs). The results of this study indicate that STING causes sterile inflammation in endothelial cells and impairs endothelial function in a diabetic mouse model. STING signaling may be a potential therapeutic target for endothelial dysfunction caused by metabolic diseases.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Animal

C57BL6/J wild-type mice were purchased from Japan SLC Inc. *Sting*$^{-/-}$ mice (C57BL6/J background) were donated by Professor Glen N. Barber of Miami University. A single dose of streptozotocin (180 mg/kg) was injected intraperitoneally in 8-week-old male wild-type and *Sting*$^{-/-}$ mice. Sex- and age-matched control mice received an injection of vehicle (0.1 mol/L sodium citrate). Some mice received an intraperitoneal injection of 1 μmol C-176 (MedKoo Biosciences Inc.), a STING inhibitor dissolved in DMSO, in 200 μL of corn oil (Sigma) 3 times per week from 3 days after streptozotocin injection. Mice were housed at a constant temperature of 23 °C and 12/12-hour dark/light cycle. Mice were euthanized by an overdose of isoflurane anesthesia (5% concentration). Animal studies conformed to the guidelines from the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the guidelines for animal experimentation at Tokushima

University. The study protocol was reviewed and approved by the Animal Care and Use Committee of Tokushima University (No. T2020-127).

Blood Glucose Level and Laboratory Data

The glucose levels of blood collected from the tail vein were measured using Startstrip XP2 (NIPRO) at 3 time points: before streptozotocin injection, 3 days after injection, and 3 weeks after injection (right before euthanization). Blood was collected from the left ventricle of the heart into EDTA-containing tubes. Plasma samples were separated from whole blood by centrifugation (7000*g*) for 15 minutes at 4 °C and kept at −80 °C until further use. Total cholesterol, high-density lipoprotein cholesterol, and triglyceride levels were measured at the Sanritsu Zelkova Examination Center (Japan).

Aorta Preparation and Vascular Reactivity Assay

Aortic samples were prepared as previously described.[21] Briefly, at the time of euthanization, the whole aorta was immediately isolated and the surrounding fat and connective tissue were carefully removed after perfusion with 0.9% sodium chloride solution via the left ventricle at constant pressure. Aortic rings (1.5–2 mm) were cut from the descending thoracic aorta for the vascular reactivity analysis. The remaining thoracic aorta and abdominal aorta were snap-frozen in liquid nitrogen for gene expression or western blotting analysis, respectively.

Vascular reactivity was examined as previously described.[21] Aortic rings were placed in organ baths filled with aerated (95% O_2 and 5% CO_2) and preheated (37 °C) modified Krebs–Henseleit buffer (118.4 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, 25 mmol/L $NaHCO_3$, 11.1 mmol/L glucose). Changes in isometric tension were recorded using a polygraph (LabChart). The viability of the aortic segments was tested with 31.4 mmol/L KCl. To determine the relaxation response, the aortic rings were contracted with phenylephrine (10^{-9} to 10^{-4} mol/L) to submaximal tension (60% of maximum). After stable contraction was determined, the rings were exposed to increasing concentrations of acetylcholine (10^{-9} to 10^{-4} mol/L) and sodium nitroprusside (10^{-9} to 10^{-4} mol/L) to obtain a cumulative concentration–response curve. In some experiments, the aortic rings were incubated with 10 μg/mL of cGAMP (InvivoGene), a specific STING agonist, for 16 hours after pretreatment with 1.0 μmol/L C-176 for 1 hour to examine the involvement of STING signaling in endothelial function. To examine the role of interferon-β in cGAMP-induced vascular dysfunction, the aortic rings were incubated with 100 ng/mL anti–interferon-β antibody (Abcam), or isotype control IgG (BioLegend) for 16 hours. To examine the effect of streptozotocin or C-176 in the in vivo condition, we collected 1 ring from 1 mouse. To examine the effect of some treatments in ex vivo experiments, we collected the required numbers of aortic rings from a single mouse and used 1 ring for each condition.

Electron Microscope Analysis

To detect the accumulation of DNA fragments in endothelial cells, immunogold staining was performed as previously described.[10] Samples were fixed in a 4% paraformaldehyde solution containing 0.1% glutaraldehyde and 0.05% Triton X-100 and then embedded in glycol methacrylate. Ultrathin (80–100 nm) sections were incubated with 1% bovine serum albumin in 0.01 mol/L PBS for 1 hour and rinsed with 0.01 mol/L PBS for 15 minutes. The sections were incubated overnight with anti–single-stranded DNA (ssDNA) antibody (Immuno-Biological Laboratories Co., Ltd.) at 4 °C, followed by incubation with 15-nm gold-labeled goat anti-rabbit IgG (BBInternational) for 2 hours at room temperature. The sections were counterstained with uranyl acetate and Reynold's lead citrate, and examined under an electron microscope (H-7600, Hitachi). As negative controls for immunohistochemical procedures, we substituted the primary antibody with an identical concentration of nonimmune IgG and performed direct incubation in colloidal gold without the primary antibody.

Cell Culture

For all in vitro experiments, we used HUVECs purchased from Life Technologies and cultured in EGM-2 (Lonza). HUVECs (passages 4–6) were treated with 10 μg/mL cGAMP.[22–24] HUVECs were also stimulated with mitochondrial DNA (mtDNA, 200 ng) using Lipofectamine TLX with Plus™ reagent (Thermo Fisher Scientific) following the manufacturer's instructions.[17] In this study, we used mtDNA extracted from THP-1 cells by using a commercially available kit (mtDNA Extractor CT kit, Fuji Film). To inhibit STING signaling, cells were pretreated for 1 hour with C-176 (1.0 μmol/L). Cells were stimulated with cGAMP or mtDNA for 24 hours to examine gene expression, 1 hour to examine cell signaling, and 24 hours to measure interferon-β secretion. Interferon-β levels were measured in the cell culture supernatant using a commercially available ELISA kit (R&D Systems).

Quantitative Polymerase Chain Reaction Analysis

RNA was extracted from cells or tissues using the NucleoSpin RNA kit (Takara Bio). cDNA was synthesized using a QuantiTecht Reverse Transcription Kit

(Qiagen). A StepOne Real-Time PCR System (Applied Biosystems) and Power SYBR Green PCR master mix (Applied Biosystems) were used for quantitative polymerase chain reaction. Primer sequences are summarized in Table S1. β-Actin and GAPDH were used as reference genes for mouse and human samples, respectively.

Western Blotting Analysis

Proteins were extracted from aortic tissue or cells using RIPA buffer (Wako Pure Chemical Industries Ltd.) containing a protease inhibitor cocktail (Roche) and phosphatase inhibitors (Roche). Proteins were separated by SDS–polyacrylamide gel electrophoresis as previously described.[21] The following primary antibodies were used: anti-STING, anti-γH2AX, anti-H2AX, anti-phosphorylated IRF3, anti-IRF3, anti-phosphorylated TANK-binding kinase 1 (TBK1), anti-TBK1 (Cell Signaling), anti-phosphorylated-eNOS^{Ser1177}, anti-eNOS (BD Biosciences), and anti-β-actin (Sigma). β-Actin was used as an internal control. Protein expression levels were analyzed with ECL-plus reagent (GE Healthcare) using a luminescent image analyzer (LAS-4000 mini, FUJIFILM).

Statistical Analysis

All numerical values are expressed as the mean±SEM. The Shapiro–Wilk normality test was used to assess the distribution of the data. All statistical data were tested for normal distribution and homogeneity. The parametric test was conducted if the data conformed to equal variance and normal distribution; otherwise, we performed the nonparametric test. Briefly, for the comparisons of the 2 groups, an unpaired 2-tailed Student's *t* test using (when appropriate) Welch's correction or the Mann–Whitney test was used. For comparisons of multiple groups, ANOVA followed by Dunnett's multiple comparison test or Welch ANOVA followed by Tamhane's T2 analysis test was used. Comparison of the dose–response curves was performed using a 2-factor repeated measures ANOVA, followed by Dunn's post hoc test. Statistical significance was set at $P<0.05$.

RESULTS

Induction of Metabolic Disorders Promotes STING Expression and DNA Damage in Aorta

Injection of streptozotocin to wild-type mice significantly elevated blood glucose, which were accompanied with the elevation of lipid levels as previously reported.[21,25] Streptozotocin injection increased the expression of STING in the aorta at both the gene ($P<0.05$) and protein levels ($P<0.05$; Figure 1A and 1B). It also tended to increase the expression of phosphorylated H2AX ($P=0.06$), also known as γH2AX, and ratio of phosphorylated H2AX to total H2AX ($P<0.05$), which are DNA damage markers in the aorta (Figure 1C). The results of immune-electron microscopy demonstrated the accumulation of single-stranded DNA in the cytosol of aortic endothelial cells in mice treated with streptozotocin (Figure 1D). These results suggest an increase in DNA damage and STING expression in the vascular system of streptozotocin-induced diabetic mice.

Genetic Deletion of STING Ameliorates Endothelial Dysfunction in Metabolic Disorders

We next investigated the role of STING in the development of endothelial dysfunction. There were no significant differences in blood glucose and lipid levels between wild-type and *Sting*$^{-/-}$ mice after streptozotocin injection (Table 1). Streptozotocin-injection impaired endothelial function in wild-type mice ($P<0.01$), as determined by the response to acetylcholine, whereas genetic deletion of STING ameliorated endothelial dysfunction caused by streptozotocin injection ($P<0.001$; Figure 2A). In contrast, no difference was observed in the endothelium-independent vascular response to sodium nitroprusside (Figure 2B). Western blot analysis of the abdominal aorta showed that the phosphorylation of eNOS^{Ser1177} was significantly higher in *Sting*$^{-/-}$ mice than in wild-type mice after streptozotocin injection ($P<0.05$; Figure 2C), supporting the results of the vascular reactivity assay that used the aortic segments obtained from diabetic mice. In this study, total eNOS expression did not differ (Figure S1A). These results indicate that STING plays a pivotal role in the regulation of endothelial function in streptozotocin-induced hyperglycemic mice.

Activation of STING Promotes Endothelial Dysfunction

To investigate the role of STING in the development of endothelial dysfunction, we treated HUVECs with the STING agonist cGAMP and found that the RNA expression of inflammatory molecules *ICAM1*, *VCAM1*, *SELE*, and *IFNB* increased significantly (Figure 3A). cGAMP decreased the levels of eNOS phosphorylation at Ser1177 ($P<0.001$); however, it did not affect total eNOS expression in this study (Figure 3B; Figure S1B). cGAMP also increased the phosphorylation rates of TBK1 and IRF3 ($P<0.001$), suggesting the activation of STING signaling in endothelial cells (Figure 3B). The phosphorylation of eNOS negatively correlated with STING downstream signaling activation (Figure S2A).

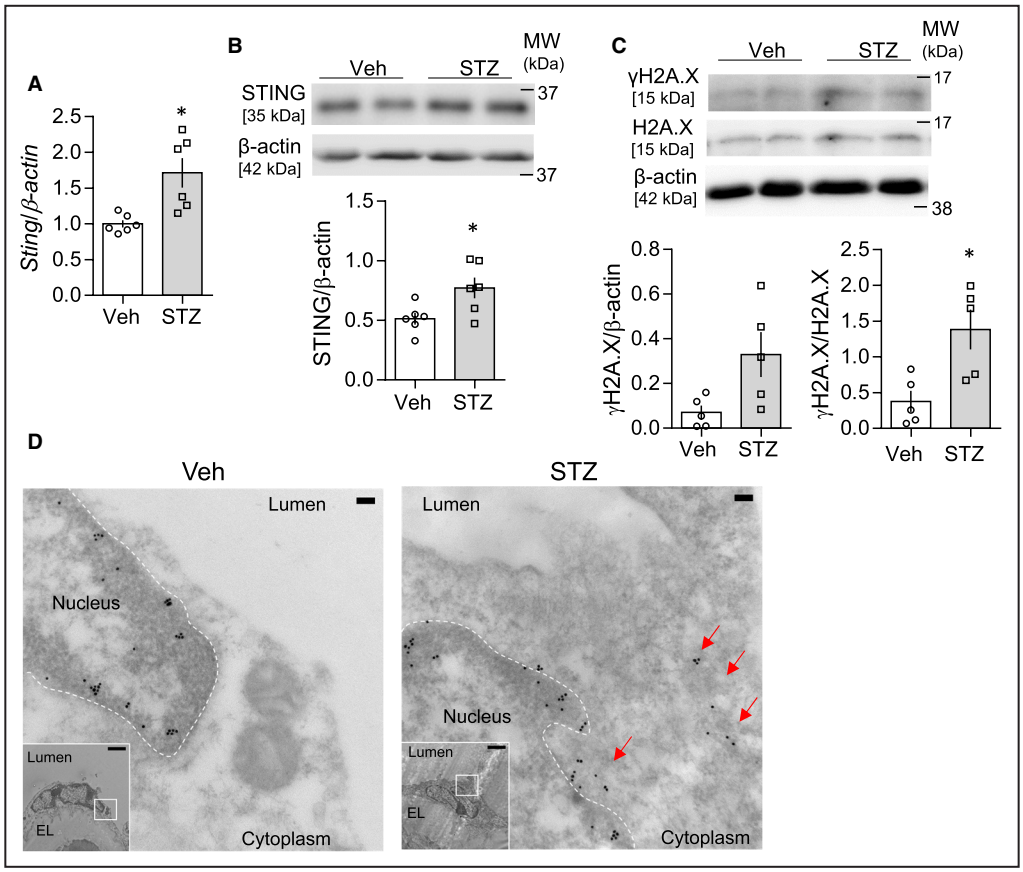

Figure 1. Streptozotocin injection promotes expression of STING in aorta.
Results of qPCR (**A**) and western blotting (**B**) showed that streptozotocin injection promoted STING expression in the aorta of wild-type mice (6 mice per group). **C**, Western blotting demonstrated an increase in the expression of γH2AX and ratio of phosphorylated H2AX to total H2AX in the aorta of streptozotocin-injected mice compared with the control mice (5 mice per group). **D**, Representative immunogold staining against single-stranded DNA showed accumulation of gold particles (15 nm, representing single-stranded DNA) in the cytoplasm of the aortic endothelial cells (red arrows) of streptozotocin-injected mice but not in those of vehicle-treated mice (3 mice per group, scale bar: 100 nm). Endothelial cells are determined morphologically, as they are a single layer of cells located on the topmost surface of the luminal side. Inset: lower magnification (scale bar: 2 μm). *P<0.05; **P<0.01 vs Veh. All values are presented as the mean±SEM. EL indicates elastic lamina; MW, molecular weight; qPCR, quantitative polymerase chain reaction; STING, stimulator of interferon genes; STZ, streptozotocin; Veh, vehicle; and WT, wild-type.

Treatment with C-176 suppressed the expression of inflammatory molecules in cGAMP-treated HUVECs (Figure 3C). Furthermore, the ELISA results demonstrated that STING inhibition by C-176 ameliorated the secretion of interferon-β from HUVECs stimulated with cGAMP (P<0.001; Figure 3D). These results indicate that cGAMP reduces eNOS phosphorylation and promotes the expression of inflammatory molecules in HUVECs, suggesting that STING activation with its agonist induces endothelial dysfunction.

Next, we treated HUVECs with DNA fragments as a stimulus for cGAMP generation. In addition to cGAMP treatment, mtDNA significantly promoted the expression of inflammatory molecules in HUVECs (Figure 4A).

Table 1. Effects of STING Deletion on Metabolic Parameters

	WT+vehicle	WT+streptozotocin	*Sting*$^{-/-}$+vehicle	*Sting*$^{-/-}$+streptozotocin
Blood glucose, mg/dL	154.9±8.1	458.1±38.8*	147.2±5.5	463.2±38.0†
Total cholesterol, mg/dL	106.1±2.8	393.5±30.7*	108.2±3.2	539.4±110.1†
Triglyceride, mg/dL	105.4±13.6	229.7±106.6	84.3±4.3	182.6±68.1
HDL cholesterol, mg/dL	63.0±1.6	148.6±9.1*	69.1±2.1	128.2±7.1†

All values are mean±SEM. *P<0.001 vs WT+vehicle and †P<0.001 vs *Sting*$^{-/-}$+vehicle. HDL indicates high-density lipoprotein; STING, stimulator of interferon genes; and WT, wild-type.

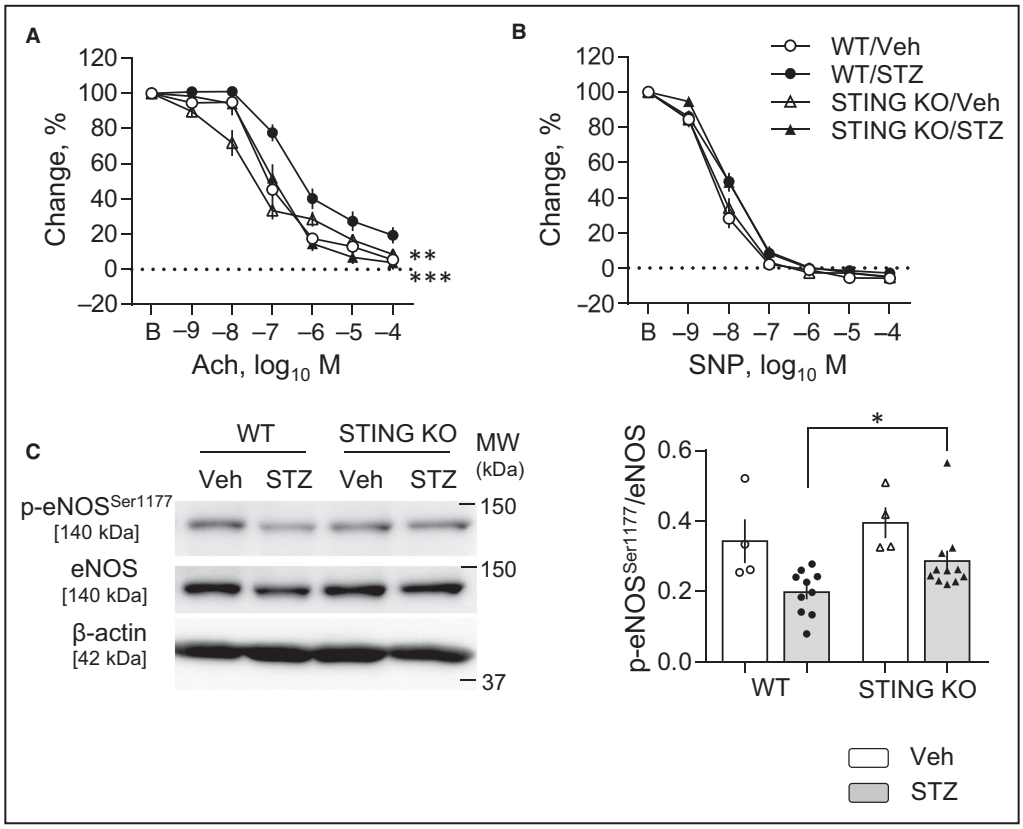

Figure 2. Genetic deletion of STING ameliorates endothelial dysfunction under metabolic stress. **A**, Genetic deletion of STING ameliorated endothelial dysfunction in metabolic disorders caused by streptozotocin injection. **B**, Endothelium-independent vasodilation in response to SNP did not differ (9–15 mice per group). ***P*<0.01; ****P*<0.001 vs WT/STZ. (**C**) eNOS phosphorylation at Ser1177 in the aorta was preserved in *Sting*$^{-/-}$ mice compared with wild-type mice, which received streptozotocin injection (4–11 mice per group). **P*<0.05. All values are presented as the mean±SEM. Ach indicates acetylcholine; eNOS, endothelial nitric oxide synthase; KO, knock-out; MW, molecular weight; SNP, sodium nitroprusside; STING, stimulator of interferon genes; STZ, streptozotocin; Veh, vehicle; and WT, wild-type.

mtDNA decreased eNOS phosphorylation levels at Ser1177 (*P*<0.05); however, it did not affect total eNOS expression in this study (Figure 4B; Figure S1C). mtDNA also promoted the phosphorylation rates of TBK1 and IRF3 (*P*<0.05), suggesting activation of STING signaling (Figure 4B). The phosphorylation of eNOS negatively correlated with STING downstream signaling activation (Figure S2B). C-176 suppressed the expression of inflammatory molecules in mtDNA-treated HUVECs, similar to the results for cGAMP-treated HUVECs (Figure 4C and 4D). These results demonstrated that exogenous DNA fragments reduce eNOS phosphorylation and promote inflammatory molecule expression in HUVECs, indicating that STING activation with mtDNA induces at least partial endothelial dysfunction.

Activation of STING Impairs Endothelium-Dependent Vascular Relaxation

We confirmed the effect of STING activation on vascular response in ex vivo experiments. Incubation with cGAMP significantly impaired endothelium-dependent vascular relaxation in aortic rings obtained from wild-type mice, but not in those from *Sting*$^{-/-}$ mice (*P*<0.001; Figure 5A). cGAMP did not affect the endothelium-independent vascular response (Figure 5B). Impairment of endothelial function by cGAMP was ameliorated in the presence of C-176 (*P*<0.05; Figure 5C and 5D). Given that STING activation promoted *IFN-β* gene and protein expression in HUVECs, we examined the effect of interferon-β neutralization and found that anti–interferon-β antibody significantly ameliorated cGAMP-induced endothelial dysfunction compared with the isotype control immunoglobulin (*P*<0.05; Figure 5E and 5F). These results suggest that STING activation partially impairs endothelial function.

Pharmacological Blockade of STING Signaling Ameliorates Endothelial Dysfunction in Diabetic Mice

To clarify the therapeutic potential of STING signaling in endothelial dysfunction, we treated wild-type mice which received streptozotocin-injection with

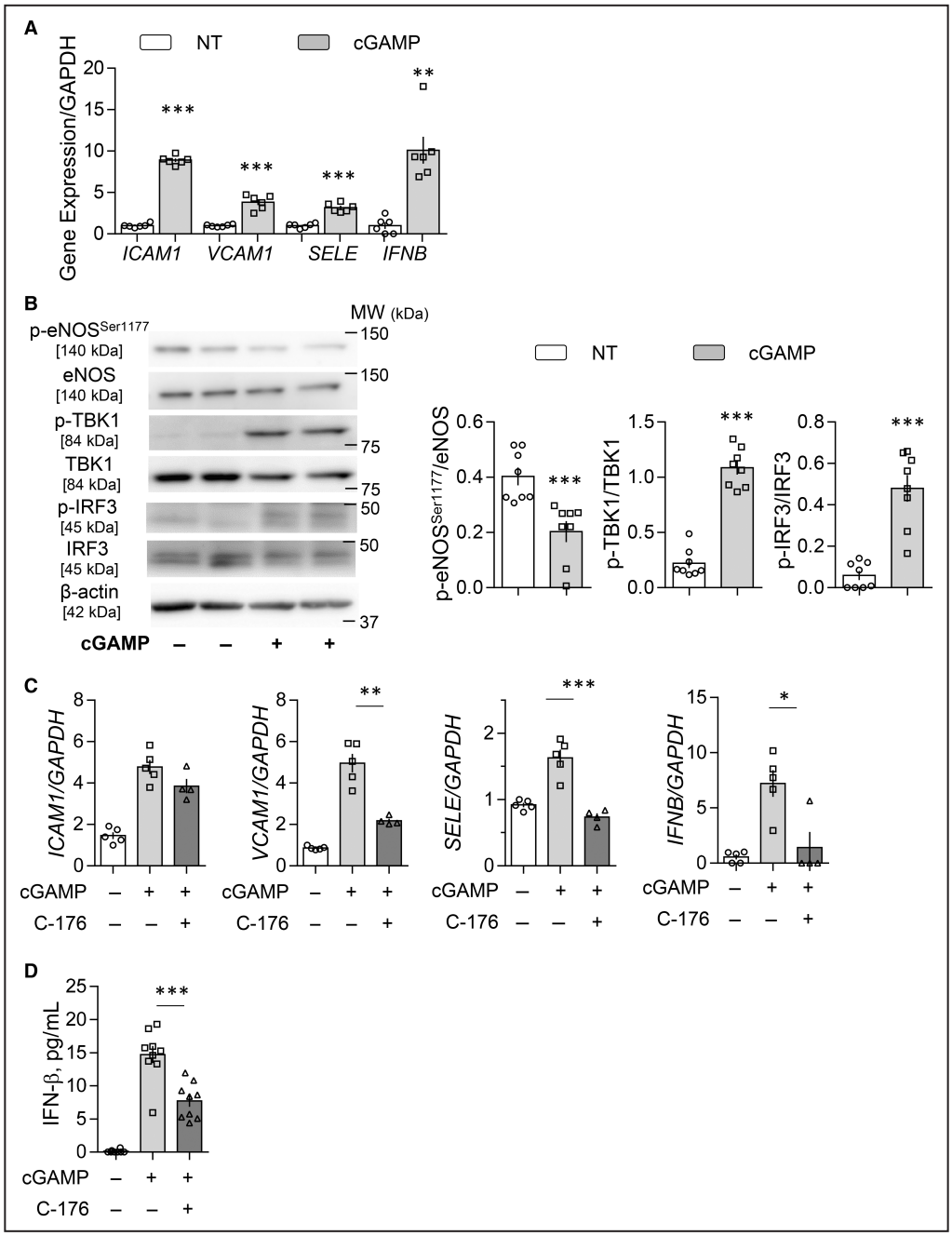

Figure 3. STING activation by cGAMP promotes inflammatory activation of endothelial cells.
A, qPCR analysis indicated that cGAMP promoted the expression of inflammatory molecules in HUVECs (n=6). ***P*<0.01; ****P*<0.001 vs NT. **B**, Western blotting showed that cGAMP significantly reduced the phosphorylation of eNOS at Ser1177, while promoting the phosphorylation of TBK1 and IRF3 compared with the nontreatment group (n=8). ****P*<0.001 vs NT. **C**, qPCR analysis indicated that C-176 ameliorated the expression of inflammatory molecules, which was promoted by cGAMP (n=4–5). **P*<0.05; ***P*<0.01; ****P*<0.001. **D**, ELISA results demonstrated that C-176 ameliorated the secretion of interferon-β from HUVECs stimulated with cGAMP (n=8–9). ****P*<0.001. All values are presented as the mean±SEM. cGAMP indicates cyclic GMP-AMP; ELISA, enzyme-linked immunosorbent assay; eNOS, endothelial nitric oxide synthase; HUVECs, human umbilical vein endothelial cells; ICAM1, intercellular adhesion molecule 1; IFN, interferon; IRF3, interferon regulatory factor 3; MW, molecular weight; NT, nontreatment; qPCR, quantitative polymerase chain reaction; SELE, e-selectin; TBK1, TANK-binding kinase 1; and VCAM1, vascular cell adhesion molecule 1.

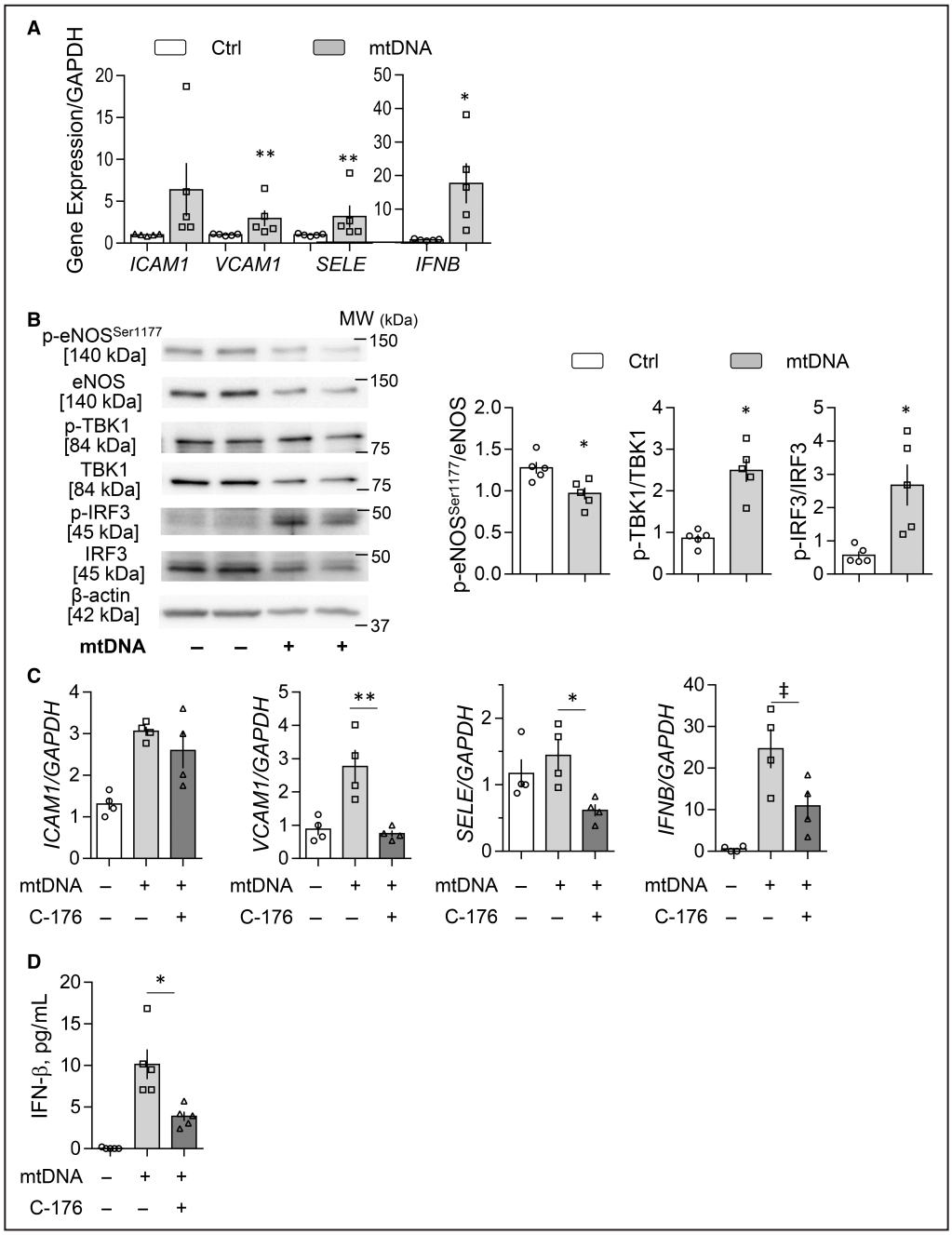

Figure 4. STING activation by DNA fragments promotes endothelial dysfunction.
A, qPCR analysis indicated that mtDNA promoted the expression of inflammatory molecules in HUVECs (n=5). **P*<0.05; ***P*<0.01 vs Ctrl. **B**, Western blotting showed that mtDNA significantly reduced the phosphorylation of eNOS at Ser1177, while promoting the phosphorylation of TBK1 and IRF3 compared with the nontreatment group (n=5). **P*<0.05 vs Ctrl. **C**, qPCR analysis indicated that C-176 ameliorated the expression of inflammatory molecules, which was promoted by mtDNA (n=4). **P*<0.05; ***P*<0.01; ‡*P*=0.05. **D**, ELISA results demonstrated that C-176 ameliorated the secretion of IFN-β from HUVECs in response to mtDNA (n=5). **P*<0.05. All values are presented as the mean±SEM. Ctrl indicates control; ELISA, enzyme-linked immunosorbent assay; eNOS, endothelial nitric oxide synthase; ICAM1, intercellular adhesion molecule 1; IFN, interferon; IRF3, interferon regulatory factor 3; mtDNA, mitochondrial DNA; MW, molecular weight; qPCR, quantitative polymerase chain reaction; SELE, e-selectin; TBK1, TANK-binding kinase 1; and VCAM1, vascular cell adhesion molecule 1.

C-176. Metabolic parameters, such as blood glucose and lipid levels, did not differ between the treated and nontreated mice (Table 2). Pharmacological blockade of STING signaling inhibited the development of endothelial dysfunction ($P<0.01$), but did not affect endothelium-independent vascular relaxation

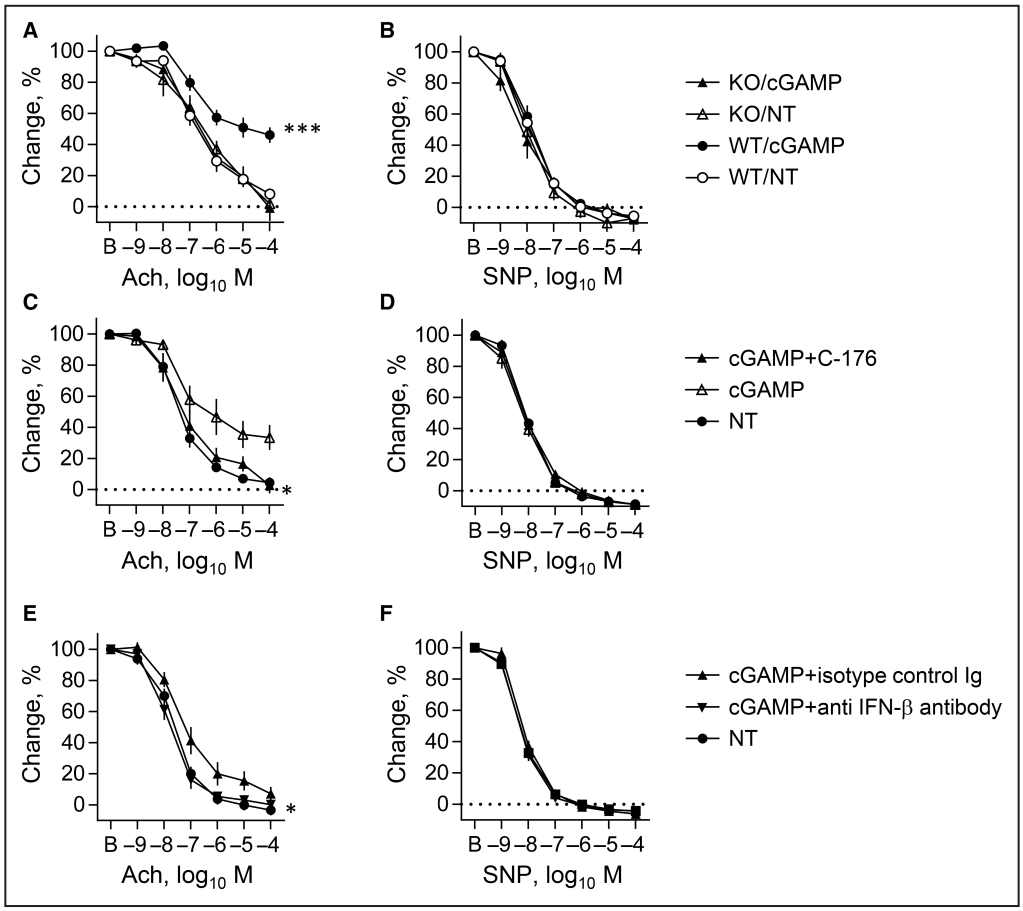

Figure 5. Activation of STING impairs endothelium-dependent vascular relaxation.
A and **B**, Vascular reactivity in response to Ach or SNP was examined using aortic rings isolated from wild-type or *Sting*$^{-/-}$ mice. **A**, cGAMP impaired the endothelium-dependent vascular response in the aortic rings obtained from wild-type mice but not in those from *Sting*$^{-/-}$ mice. **B**, cGAMP did not affect the endothelium-independent vascular response in either wild-type or *Sting*$^{-/-}$ mice. Wild-type mice: 7 mice per group, *Sting*$^{-/-}$ mice: 5 mice per group. Two rings were collected from each mouse, and 1 ring was used for each condition. ***P<0.001 vs WT/NT. **C** and **D**, The effect of C-176 on vascular reactivity was examined in the aortic rings obtained from wild-type mice. **C**, C-176 ameliorated endothelial dysfunction induced by cGAMP. **D**, C-176 did not affect the endothelial-independent vascular response. Nine mice per group. Three rings were collected from each mouse, and 1 ring was used for each condition. *P<0.05 vs cGAMP+C-176. **E** and **F**, The effect of neutralizing anti–interferon-β antibody on vascular reactivity was examined in the aortic rings obtained from wild-type mice that had been treated with cGAMP. **E**, Anti-interferon-β antibody ameliorated cGAMP-induced endothelial dysfunction in the aortic rings. **F**, Neither cGAMP nor anti–interferon-β antibody affected the endothelial-independent vascular response. Ten mice per group. Three rings were collected from each mouse, and 1 ring was used for each condition. *P<0.05 vs cGAMP+isotype control immunoglobulin. All values are presented as the mean±SEM. Ach indicates acetylcholine; cGAMP, cyclic GMP-AMP; IFN, interferon; Ig, immunoglobulin; KO, knock-out; NT, nontreatment; SNP, sodium nitroprusside; Veh, vehicle; and WT, wild-type.

(Figure 6A and 6B). C-176 administration also restored eNOS phosphorylation at Ser1177, which was affected by streptozotocin (P<0.05; Figure 6C). There was no statistical difference in total eNOS expression among all groups (Figure S1D). These results suggest that the inhibition of STING signaling may be a potential therapeutic target for vascular complications related to diabetes.

DISCUSSION

Accumulating evidence suggests that STING signaling contributes to various inflammatory conditions.[17–19] In this study, we demonstrated that the induction of diabetes by streptozotocin injection induced DNA damage in the aorta and the accumulation of DNA fragments in ECs. We also found that STING expression was upregulated

Table 2. Effects of Pharmacological Blockade of STING on Metabolic Parameters

	Vehicle	Streptozotocin	Streptozotocin+ C-176
Blood glucose, mg/dL	130.8±7.2**	620.8±54.3	665.8±54.0
Total cholesterol, mg/dL	93.5±4.7**	207.6±23.9	206.5±22.4
Triglyceride, mg/dL	107.6±13.5*	387.3±114.1	441.3±49.6
HDL cholesterol, mg/dL	51.4±3.1**	92.8±9.0	94.0±8.1

All values are mean±SEM. *P<0.05 and **P<0.01 vs vehicle. HDL indicates high-density lipoprotein; and STING, stimulator of interferon genes.

in the aorta and that the deletion or blockade of STING ameliorated the development of endothelial dysfunction in this condition. The results of the in vitro and ex vivo experiments support a causal role for STING signaling in the impairment of EC function and indicate that the DNA damage response regulated by STING is involved, at least in part, in the pathogenesis of endothelial dysfunction caused by metabolic disorders.

Immune cells such as macrophages and dendritic cells express pattern recognition receptors and play pivotal roles in the innate immune system. However, in addition to immune cells, ECs play roles in the immune system. ECs provide a platform for leukocyte attachment and migration to vessel walls by expressing adhesion molecules and chemokines in response to various stimuli. ECs also detect exogenous pathogens in the bloodstream.[26] Recent advances in immunology have verified that, through their pattern recognition receptors, ECs detect endogenous danger signals released by metabolic abnormalities or aging-related factors.[27] For example, the detection of polysaturated fatty acids by Toll-like receptors 2 and 4 induces proinflammatory responses in ECs.[28] ECs also have other sensing mechanisms for damage-associated molecular patterns and pathogen-associated molecular patterns, which contribute to various pathophysiological conditions.[29]

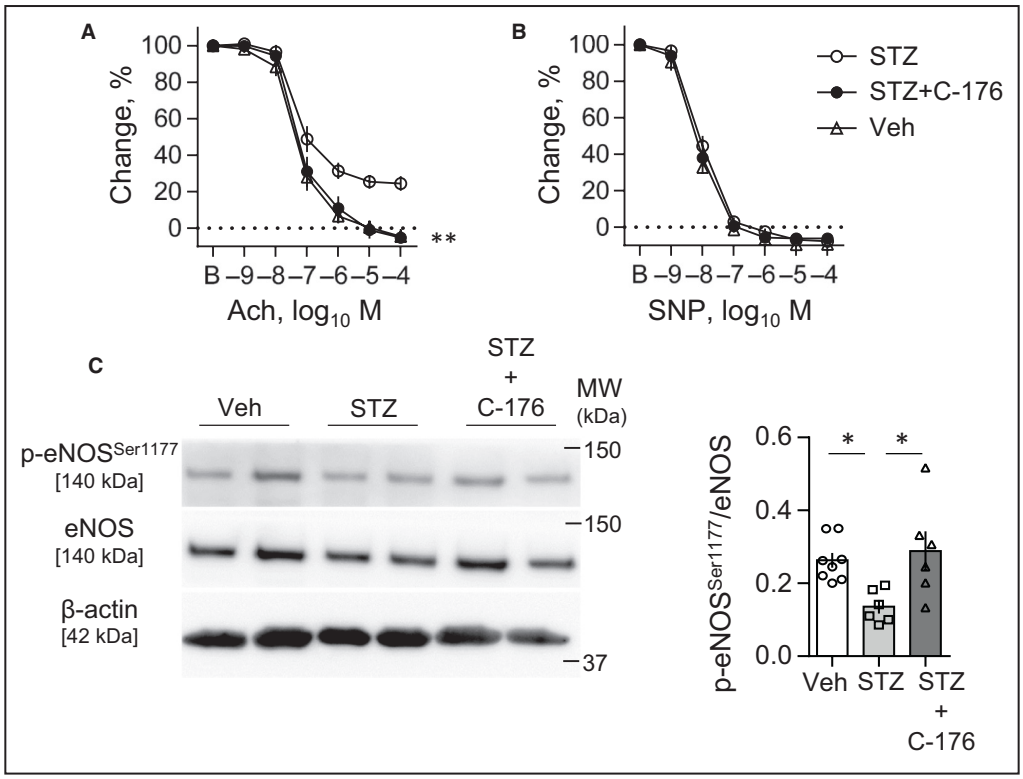

Figure 6. Pharmacological blockade of STING ameliorates endothelial dysfunction caused by metabolic disorders.
A and **B**, To examine the effect of the pharmacological blockade of STING on endothelial function, vascular reactivity in response to acetylcholine or SNP was examined using aortic rings isolated from wild-type mice (8 mice per group). **A**, Administration of C-176 ameliorated endothelium-dependent vasodilation in wild-type mice that received streptozotocin injection. **B**, Endothelial-independent vasodilation did not differ among the 3 groups. **P<0.01 vs STZ. **C**, Induction of metabolic stress reduced eNOS phosphorylation at Ser1177; however, C-176 ameliorated this response (6–8 mice per group). *P<0.05. All values are presented as the mean±SEM. Ach indicates acetylcholine; eNOS, endothelial nitric oxide synthase; MW, molecular weight; SNP, sodium nitroprusside; STZ, streptozotocin; and Veh, vehicle.

Metabolic stress-induced DNA damage, which is regulated through several molecular mechanisms, is associated with the pathogenesis of cardiometabolic disorders. The DNA damage response in subjects with diabetes has been extensively investigated. mtDNA damage is an underlying mechanism of vascular dysfunction and atherogenesis in patients with diabetes.[6] Several clinical studies have reported that oxidative DNA damage is independently associated with cardiovascular death in type 2 diabetes.[30] We and others have also demonstrated the participation of DNA damage response in vascular complications associated with dyslipidemia.[8–10] Given the importance of DNA sensors in the regulation of the immune response, several recent studies have implicated STING in the pathogenesis of various inflammatory diseases, including atherosclerosis and metabolic diseases. In this study, we found that the induction of metabolic stress promoted STING expression in the aorta and that STING signaling impaired endothelial function, as determined by the expression of inflammatory molecules and acetylcholine-dependent vascular relaxation. In our in vitro studies, the STING agonist cGAMP clearly reduced eNOS phosphorylation in HUVECs. cGAMP activated the TBK1-IRF3 pathway, a downstream signal of STING signaling, leading to increased expression of inflammatory molecules, such as *VCAM1* and *IFNB*. Similarly, mtDNA, which stimulates cGAMP production in the cytosol, activated the TBK1-IRF3 pathway and increased the levels of inflammatory molecules in HUVECs. These responses were attenuated by the STING inhibitor C-176, suggesting that mtDNA induces endothelial dysfunction via STING signaling. In the ex vivo experiments, STING activation with cGAMP impaired endothelial function, which was attenuated by the STING inhibitor and neutralizing anti–interferon-β antibody. These results support the causal role of STING signaling in endothelial dysfunction associated with metabolic disorders and identify STING as a potential therapeutic target in such condition.

Recent studies have also shown the contribution of STING signaling in the regulation of endothelial function. These studies demonstrated that the activation of STING signaling pathway by either cellular aging or palmitic acid decreased total eNOS expression in ECs, resulted in endothelial dysfunction.[31,32] In our study, STING activation did not affect total eNOS expression, although it reduced eNOS phosphorylation. In this study, a neutralizing antibody for interferon-β ameliorated cGAMP-induced endothelial dysfunction in diabetic condition. Previous studies reported that STING activation reduced eNOS expression associated with senescence or endothelial-to-mesenchymal transition. Taken together, inflammatory responses caused by STING signaling contributes to the development of endothelial dysfunction, although further studies are needed to clarify the mechanism by which STING regulates EC function.

Endothelial dysfunction, which initiates atherosclerosis, is one of the therapeutic targets for vascular diseases; however, the development of therapeutic strategies that aim to ameliorate endothelial dysfunction has not been enough. In this study, we demonstrated that the pharmacological blockade of STING signaling by C-176 ameliorated the development of endothelial dysfunction in streptozotocin-induced diabetic mice. Our data suggest that STING may be a therapeutic target against vascular dysfunction associated with metabolic disorders. However, DNA sensors, such as STING, are integral to maintaining homeostasis and therefore further studies are required to safely target STING. Recent studies have shown that the innate immune system contributes to the development of inflammatory diseases.[33] DNA fragments, especially those from exogenous organisms, function as inflammatory stimuli for DNA sensors and activate the immune system. DNA fragments released from host cells trigger inflammatory responses in a similar manner. The triggers that stimulate the release of endogenous DNA fragments are not fully understood. Severe physical trauma, for example, can increase the release of DNA fragments[34,35]; however, recent studies suggest that overnutrition or aging-related factors also result in the release of self-derived DNA fragments.[17–19] In this study, induction of diabetes led to an increase in DNA damage markers in the aorta and the accumulation of DNA fragments in ECs. Therefore, developing the novel strategy targeting the innate immune system is important, although the effective treatment of lifestyle-related diseases, such as diabetes, by a combination of medical treatment and a healthy lifestyle, is especially required to reduce the adverse responses of the innate immune system to aberrant DNA damage or sensing mechanisms.

We acknowledge some limitations to our study. First, we used streptozotocin injection in this study. Although streptozotocin is commonly used to induce diabetes, this model does not completely represent type 2 diabetes, and the results from this model are therefore not directly interpretable to humans. Also, similar to previous studies,[21,25] streptozotocin injection elevated lipid levels compared with the control group. Dyslipidemia is also suggested to increase DNA damage and STING activation.[8] Therefore, in this study, STING activation observed in streptozotocin-injected mice might be caused by dyslipidemia partially, in addition to hyperglycemia. However, there were no significant differences in these metabolic parameters between wild-type and *Sting*$^{-/-}$ mice. Second, we used a global STING knockout model. Recent studies showed the expression and function of STING in multiple cell types. Therefore, STING in other cell

types might contribute to vascular function. In this study, in vitro experiments clearly demonstrated that STING activation impairs endothelial function; however, a cell-type–specific STING expression or deletion model would be more informative. Third, recent studies suggested the crosstalk between multiple signal pathways related to innate immune systems. For instance, cGAMP may activate NLRP3 inflammasome.[36] The role of the innate immune system in the pathogenesis of endothelial dysfunction associated with metabolic abnormalities might be more complicated. In addition, in this study, we could not detect the expression of *IFNA*, another type I interferon, in ECs. Previous studies suggested that STING activation induces a predominant interferon-β expression compared with interferon-α in ECs.[20,37] Other studies reported that the response to STING signaling is different between both cell types and time points after the stimulation.[38,39] Thus, further studies are needed to clarify the role of STING signaling in the regulation of endothelial function under diabetic conditions and to develop therapeutic strategies for endothelial dysfunction targeting STING.

In conclusion, DNA damage and subsequent STING activation at least partially contribute to the development of endothelial dysfunction associated with metabolic disorders. Our findings provide new insights into the pathogenesis of vascular inflammation and endothelial dysfunction. The innate immune system, including STING signaling, may be a potential therapeutic target against vascular complications associated with metabolic disorders.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Table S1
Figures S1–S2

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