RESEARCH

ERK-containing microparticles from a diabetic mouse induce endothelial dysfunction

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Abstract

Endothelial dysfunction is a hallmark of diabetic vascular complications. Microparticles (MPs) are small vesicles shed from the surface of blood and vascular cells that act as stimuli and during apoptosis. Circulating MPs of diabetic rats have been shown to induce endothelial dysfunction. However, the underlying mechanisms require further study. In this study, we investigated how MPs from diabetic mice affect endothelial function. MPs were collected from streptozotocin-induced diabetic mice and Institute of Cancer Research (ICR) mice as controls. The levels of MPs were assessed and characterized by flow cytometry, enzyme-linked immunosorbent assay and dot blotting. Normal mice aortas were incubated with MPs and expressions of enzymes and vascular relaxation were analyzed. We found that (1) circulating MPs level increased in diabetic mice; (2) MPs impaired endothelial-dependent relaxation in mice aorta, but diabetic mice-derived MPs (diabetes mellitus (DM) MPs) were easier to attach to the endothelial cells than were control MPs; (3) DM MPs had more extracellular signal-regulated kinase (ERK)1/2 than did control mice-derived MPs, and they induced ERK1/2 activation in mice aortas; (4) DM MPs decreased endothelial nitric oxide synthase (eNOS) in mice aortas, and eNOS was emitted from endothelial cells to blood in the shape of endothelial MPs. DM MPs significantly altered endothelial function by activation of ERK1/2, which might provide a therapeutic target for diabetic vascular complications.

Key Words

- diabetic mouse
- microparticles
- ERK1/2
- eNOS
- NO

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Introduction

Vascular complications, which include cardiovascular diseases and atherosclerosis, are the leading cause of morbidity and mortality in diabetes mellitus (DM) (Standl *et al.* 1996, Schram *et al.* 2002). Although the mechanisms by which DM increases vascular complications are incompletely understood, strong supportive evidence from experimental and clinical studies points to vascular endothelial dysfunction as a critical inducer (Lockhart *et al.* 2008). Endothelial dysfunction is characterized by a decrease in production of nitric oxide (NO). NO is a gaseous molecule constitutively produced by endothelial NO synthase (eNOS) in endothelial cells that induces the vascular relaxation (Elrod *et al.* 2006, Ishida *et al.* 2014, 2016, Taguchi *et al.* 2017*a*,*b*, 2018). Furthermore, impairment of endothelium-derived NO is a common feature of endothelial dysfunction in diabetic individuals (Steinberg *et al.* 1996, Montagnani *et al.* 2002, Taguchi *et al.* 2016). In this study, we show the important role of the factors that contribute to diabetes-associated vascular complications, in particular, NO and microparticles (MPs).

MPs are submicron membrane vesicles released from cells that act as stimuli and during apoptosis (Leroyer *et al.* 2007, Burger *et al.* 2013). Circulating MPs are those released from various cell types, such as platelets, leukocytes, endothelial cells, erythrocytes and smooth muscle cells in the blood and from the vascular wall (Leroyer *et al.*

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2007, Shantsila *et al.* 2010, Rautou *et al.* 2011). MPs can be detected in the plasma of healthy subjects and are putative biomarkers of disease. For example, an elevation of circulating MP levels has been reported in vascular diseases, such as thrombotic diseases, diabetes and cardiovascular diseases (Leroyer *et al.* 2007, Nomura 2009, Shantsila *et al.* 2010, Burger *et al.* 2011, 2013, Rautou *et al.* 2011). Circulating MPs are considered to have a crucial role in inflammation, coagulation, endothelial function and angiogenesis. MPs are no longer taken as innocent bystanders because several studies have pointed out that MPs influence vascular homeostasis, thereby contributing to the progression of vascular diseases (Dignat-George & Boulanger 2011). Elevated numbers of MPs were found in many vascular diseases associated with endothelial dysfunction (Preston *et al.* 2003). However, the interpretation of such studies is difficult because protein fractions of MPs, and thus likely MPs effects, greatly vary depending on the stimulus initiating cell blebbing and MPs release (Huber *et al.* 2002, Bernimoulin *et al.* 2009).

MPs found in blood are important elements that regulate cellular interactions under both physiological and pathological conditions. They have an important role in blood clot formation and increased endothelial dysfunction. Our previous study found that MPs from DM rats and high glucose/angiotensin II (Ang II)-derived extracellular vesicles (as known MPs) from human umbilical vein endothelial cell (HUVECs) inhibited the acetylcholine (ACh)-induced endothelial-dependent relaxation response and increased the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) (Ishida *et al.* 2016, Taguchi *et al.* 2017*a*,*b*). This suggests that ERK1/2 is a strong candidate for mediation of endothelial dysfunction induced by MPs from DM. Therefore, in the present study, we determined the effects of circulating MPs derived from well-established streptozotocin (STZ)-induced DM model mice (DM MPs) on endothelial function and explored whether ERK1/2 is involved in the mechanism of DM-derived endothelial dysfunction. Furthermore, little has been known about the components of the MPs and the underlying mechanism of action in the past, so we extended the investigation to MPs components from DM mice.

Materials and methods

Animals and the STZ-induced diabetic mouse model

Male ICR mice (4 weeks) were obtained from the Tokyo Animal Laboratories (Tokyo, Japan) and housed under 12-h light/12-h darkness with free access to water and food. One week later, the mice were randomly divided into two groups: the control group and the STZ-treated group. Diabetes was induced by a single tail vein injection (200 mg/kg) of STZ (Wako Chemical) dissolved in a citric acid buffer, as reported previously (Taguchi *et al.* 2016). Control mice were administered the same volume of a citric acid buffer alone. The plasma glucose level was measured 4 months later to confirm diabetes (blood glucose >400 mg/dL). The animals were killed at 12–15 weeks after injection, the arterial blood was obtained, and the thoracic aortas were removed from the control mice. All experiments were performed in accordance with the guidelines of and approved by the Hoshi University Animal Care and Use Committee (accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan).

Measurement of plasma glucose

Plasma samples have been described in previous publications from our laboratory (Ishida *et al.* 2014, 2016, Taguchi *et al.* 2016, 2017*a*, 2018). Glucose concentration was determined using a commercially available enzyme kit (Wako Chemical).

MPs isolation

The MPs isolation protocol used was adapted from those previously published by our laboratory (Ishida *et al.* 2016). In short, a total of 2 mL of blood was collected via the abdominal aorta from the Control or DM mice and mixed with sodium citrate anti-coagulant. The mixture was then centrifuged at 5000***g*** for 10 min to remove platelets. A total of 500 µL of the platelet-free plasma supernatant containing the MPs was centrifugated at 30,000***g*** for 2 h to eliminate plasma and to pellet MPs. The supernatant was replaced with 100 µL of phosphate buffered saline. In this study, in diabetic mice, the aortic rings were treated with an excessive amount of MPs. However, this concentration is similar to pathophysiological conditions.

Measurement of vascular reactivity

The measurement of vascular reactivity in mouse thoracic aortas has been described previously (Ishida *et al.* 2014, 2016, Taguchi *et al.* 2016, 2017*a*,*b*, 2018). In brief, 12–15 weeks after STZ injection, the mice were killed by decapitation under anesthesia. The thoracic aortas were then gently isolated and transferred to Krebs–Henseleit solution (KHS; consisting (in mmol/L) of 118.0 NaCl,

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4.7 KCl, 25 $NaHCO_3$, 1.8 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgSO_4$, and 11.0 glucose) that was aerated with carbogen (95% O_2, 5% CO_2). The adherent tissues were immediately resected from the aortas and then cut into 2 mm long circular segments, which were suspended from a pair of stainless-steel pins containing 10 mL of KHS. We adjusted the resting tension to 1.5 g for aortas. To examine vascular relaxation in response to ACh (10^{-9} to 10^{-5} mol/L) and sodium nitroprusside (SNP; 10^{-10} to 10^{-5} mol/L), the aortas were precontracted submaximally (1.0 ***g*** force) using the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) at concentrations ranging from 10^{-6} to 3×10^{-6} mol/L. For the vascular relaxation responses, the results were expressed as percent relaxation (% of induced tone), with 100% representing the difference between the resting value under basal conditions and the constricted value in response to $PGF_{2\alpha}$. SNP (a NO donor) was used as an endothelium-independent vascular relaxation response. When necessary, the aortas were preincubated for 30 min with N^G-nitro-L-arginine (L-NNA), as indicated before the initiation of the reaction. Moreover, ACh and SNP responses were also evaluated after incubation with vehicle, control mice-derived MPs (Control MPs) or DM MPs (respectively 50 µL; as above) for 30 min. To confirm the possibility of the adhesion of MPs to aortas, concentration–response curves for ACh and SNP were performed once again after washing out.

Immunofluorescent analysis

In order to test the hypothesis of the adhesion of DM MPs to aortas, MPs (10,000 MPs/µL) were incubated overnight with DyLight 488-conjugated anti-mouse CD42b antibody (1:200; Novus Biologicals, Cambridge, UK). Unbound antibody was removed by washing the MPs twice. These MPs were resuspended in 50 µL of PBS.

Control aortas were rapidly frozen without fixation, embedded in O.C.T. Compound (Tissue-Tek; Sakura Finetek Japan, Tokyo, Japan) and cut into 6 µm-thick cross-sections. The sections were placed on glass slides, washed with PBS, and then incubated with CD42b-labeled Control or DM MPs for 30 min at room temperature in the dark. After two more washouts to remove excess unadhered MPs, cover slips were mounted on each slide. All images were recorded using a confocal microscope (Zeiss LSM 5 Exciter; Zeiss).

Detection of MPs by using flow cytometry and ELISA

Determination of MPs was performed as described previously (Ishida *et al.* 2016). Briefly, 50 µL of MPs was incubated with FITC-labeled anti-CD42b (a platelet-derived MP (PDMP) marker; Bioss Antibodies Inc., MA, USA) for 30 min at 4°C in the dark. Samples were diluted with 1 mL of filtered phosphate buffered saline before flow cytometric analysis. We used non-stained Control MPs as the reference to discriminate true events from noise, and made the gate between the presence of MPs and the absence of those. Stained or non-stained samples were quantified by using a FACS Calibur (BD Biosciences, USA). The derivation and amounts of MPs were further confirmed by ELISA. PDMPs were measured by using a sandwich ELISA PDMP assay kit (Tanpaku Seisei Inc., Gunma, Japan).

Dot blots for MPs content organization

MPs spotted onto PVDF membranes (Bio-Rad Laboratories). These were probed with Akt (Cell Signaling Technology), eNOS (BD Bioscience), ERK1/2 (Cell Signaling Technology), p38 mitogen-activated protein kinase (MAPK; Cell Signaling Technology), Jun amino-terminal kinase (JNK; Cell Signaling Technology) and caveolin-1 (Cell Signaling Technology) antibody (1:1000) and detected using a horseradish peroxidase-conjugated secondary antibody. Experiments were repeated at least six times. Caveolin-1 was used for normalization of the results.

Measurement of vascular NOx level

The total NOx (nitrate+nitrite) level was detected in the aorta using an automated NO detector/high-performance liquid chromatography system (ENO20; Eicom, Kyoto, Japan) according to the manufacturer's protocol (Ishida *et al.* 2016, Taguchi *et al.* 2016, 2017*a*,*b*, 2018). Each aorta was cut into transverse rings of 4 mm in length. These were placed in KHS at 37°C and treated with vehicle, Control MPs or DM MPs (10 µL; as above) for 30 min, and then stimulated with ACh (10^{-6} mol/L) for 20 min. The amount of NOx was expressed as follows: non-stimulated NOx or ACh-stimulated NOx (10^{-5} mol/20 (min) g (weight of the aorta)).

Western blotting

Aorta homogenate: aorta was provided as above (measurement of NOx level section); immediately after, it was frozen in liquid nitrogen and stored in a refrigerator at −80°C as previously described (Ishida *et al.* 2014, 2016, Taguchi *et al.* 2016, 2017*a*,*b*, 2018). The aortas were macerated in liquid nitrogen and homogenized in a modified RIPA buffer (Thermo Scientific) to prevent

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proteolysis and maintain phosphorylation of proteins (protease and phosphatase inhibitor cocktail; Roche Diagnostics). Then, homogenates were centrifuged at 4°C, 16,000 ***g*** for 10 min, and protein in supernatant was quantified by the bicinchoninic acid assay with bovine serum albumin protein as a standard protein.

Western blot analysis: aortic protein extracts (25 µg) were subjected to electrophoresis on 10% polyacrylamide gel and transferred to a PVDF membrane. After the transference, membranes were blocked with a blocking reagent (ImmunoBlock; DS Pharma Biomedical Co., Ltd., Osaka, Japan) for 90 min at room temperature. Membranes were incubated overnight with the primary antibody for phosphorylated eNOS (p-eNOS (Ser 1177)) (1:1000, Cell Signaling Technology), eNOS (1:1000), phosphorylated Akt (p-Akt (Ser473)) (1:1000, Cell Signaling Technology), Akt (1:1000), phosphorylated p38 MAPK (p-p38 MAPK (Thr180/Tyr182)) (1:2000, Cell Signaling Technology), p38 MAPK (1:2000), phosphorylated p44/42 MAPK (ERK1/2) (p-ERK1/2 (Thr202/Tyr204)) (1:2000, Cell Signaling Technology), ERK1/2 (1:2000), phosphorylated JNK (p-JNK (Tyr185)) (1:1000, Cell Signaling Technology) and JNK (1:1000) at 4°C. Then, the incubation was performed with an anti-rabbit or mouse secondary antibody (1:10,000) for 20 min at room temperature. β-Actin (1:5000, Sigma Chemical Co.) was used for normalization of the results. Ratios were calculated for the optical densities of phosphorylated Akt, eNOS, p38 MAPK, ERK1/2 or JNK to those of the corresponding total protein bands.

eNOS detection in plasma and MPs

The eNOS concentrations of total and phosphorylation in plasma and MPs were determined by ELISA with a commercially available detection kit. P-eNOS (Ser1177) and total eNOS ELISA kit (RayBiotech, Norcross, GA, USA) was used. The 'control' in the vertical axis label indicates the 'control' used in the ELISA kit.

Statistical analysis

All values are given as the mean ± S.E. N is the number of animals tested. Statistical analysis was performed by using GraphPad Prism 6.0. The independent-sample *t* test, repeated-measures ANOVA and 2-way ANOVA with *post hoc* correction for Tukey multiple comparisons test were used. For ANOVA, the interaction and/or the effect of the specific treatment was analyzed. A value of $P<0.05$ was considered indicative of statistical significance.

Results

DM mice and vascular characteristics

Body weight levels were consistently lower in DM mice than in control mice. Plasma glucose levels obtained at the end of the study were significantly and comparably elevated in DM mice (Table 1).

We determined the effects of DM mice on endothelial function by measuring ACh- and SNP-induced relaxations of aortic rings. DM induced a significant reduction in ACh-induced relaxation relative to that in the control mice (Fig. 1A). Moreover, L-NNA, a NOS inhibitor, completely abolished vascular relaxation in control and DM mice. On the other hand, DM did not affect SNP-induced endothelium-independent relaxation compared with control mice (Fig. 1B). These data indicate that the relaxation is fully dependent on NO in these mice and confirms that DM induces endothelial dysfunction by reducing NO bioavailability. These results are consistent with those of our previous studies (Ishida *et al.* 2014, 2016, Taguchi *et al.* 2016, 2017*b*). We thought that there were important results suggesting the possibility that MPs are involved in vascular endothelial dysfunction in DM.

Table 1 Body weight and plasma glucose levels of control mice and DM mice.

	Control mice	DM mice
Body weight (g)	62.7 ± 1.0	38.4 ± 1.4***
Glucose (mg/dL)	105.4 ± 8.1	576.0 ± 22.2***

Values are means ± S.E. ($n = 8$).
***$P < 0.001$ vs Control mice.

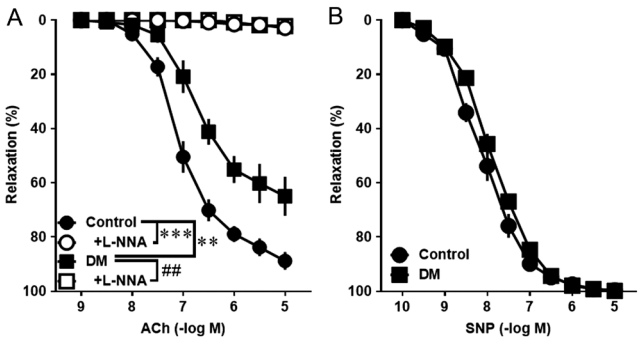

Figure 1
Relaxant effects of ACh and SNP in aortas from control and DM mice. (A) ACh-induced relaxation of PGF2α precontracted aorta from control and DM mice in the absence and presence of L-NNA (10^{-4} mol/L). (B) SNP-induced relaxation of PGF2α precontracted aorta from control and DM mice. Results are expressed as the mean ± S.E.; $n = 4–7$. **$P < 0.01$, ***$P < 0.001$ vs Control mice. ##$P < 0.01$ vs DM mice.

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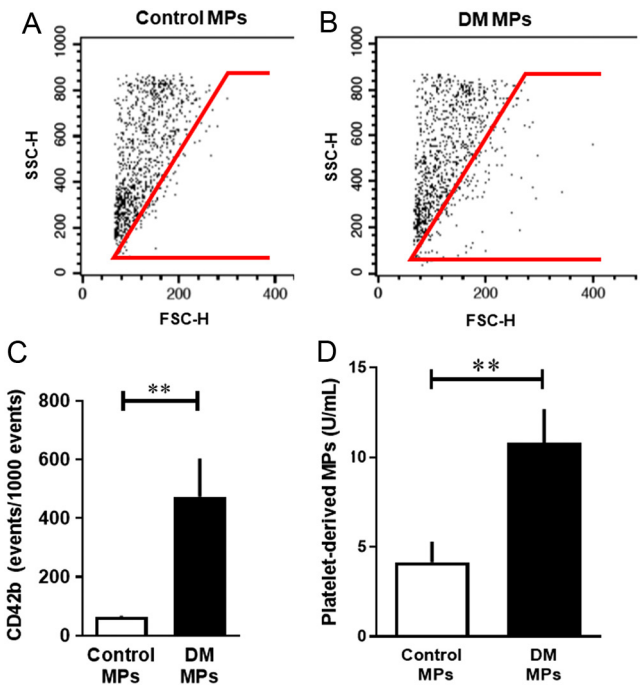

Figure 2
Flow cytometry and ELISA analysis of MPs. (A and B) Representative traces of flow cytometry for MPs from control (A) and DM mice (B). Samples were stained with CD42b-fluorescein isothiocyanate. (C) The number of CD42b-positive MPs from A or B. (D) Platelet-derived MP levels for control and DM mice were measured by using an ELISA kit. Results are expressed as the mean ± S.E.; $n = 8$. $^{**}P < 0.01$ vs Control MPs. A full colour version of this figure is available at https://doi.org/10.1530/JOE-18-0616.

We succeeded in obtaining MPs taken from a DM mouse for the first time. By using flow cytometric analysis, we found that the numbers of circulating MPs (CD42b) were significantly increased in DM mice relative to those in control mice (Fig. 2A, B and C). Further investigation revealed that DM MPs significantly increased relative to Control MPs using an ELISA and circulating MPs was due to PDMPs as shown in Fig. 2D.

DM MPs-induced endothelial dysfunction in aorta

To determine the effect of MPs on endothelial-dependent and -independent vascular relaxation, we assessed ACh- and SNP-induced relaxation in $PGF_{2\alpha}$-preconstricted normal aortic rings treated with vehicle, Control MPs and DM MPs (Fig. 3). The endothelial-dependent relaxation to ACh was significantly impaired in aorta treated with either Control MPs or DM MPs relative to aorta treated with vehicle (Fig. 3A). Predictably, we found that MPs (either Control MPs or DM MPs) did not affect the endothelial-independent response to SNP in aorta (Fig. 3B). The results suggested that MPs caused more potent impairment in vascular reactivity in a short time, but there was little difference in impairment between Control MPs and DM MPs.

To evaluate how MPs affect endothelial function, the endothelial-dependent and -independent relaxation responses were measured in aorta after washing our MPs. In this study, we called the first Ach- or SNP-induced relaxation response the 'first round' and the next response after washing the 'second round' (Fig. 4A). The endothelial-dependent relaxation response to ACh was comparable between the two groups (first round and second round) (Fig. 4B). When vehicle was treated, washing out had no effect on the second round of ACh-induced relaxation response. Moreover, although DM MPs did not affect the second round of ACh-induced relaxation, it was normalized in the aorta treated with Control MPs (Fig. 4C and D), which indicated the difference in characteristics between Control MPs and DM MPs. The SNP-induced relaxation effects in aortas were not significantly different between the first and second rounds among the vehicle, Control MPs and DM MPs mouse groups (Fig. 4E, F and G).

Furthermore, we showed that the ACh-induced relaxation response was not affected in the aortas exposed to high concentrations of Control MPs (data not shown). In addition, Tual-Chalot *et al.* (2010) reported that double the amount of Control MPs was unable to induce endothelial dysfunction. We considered that these results indicated that the effects induced by MPs are independent of their number but dependent on the different compositions and/or origin between control and DM MPs.

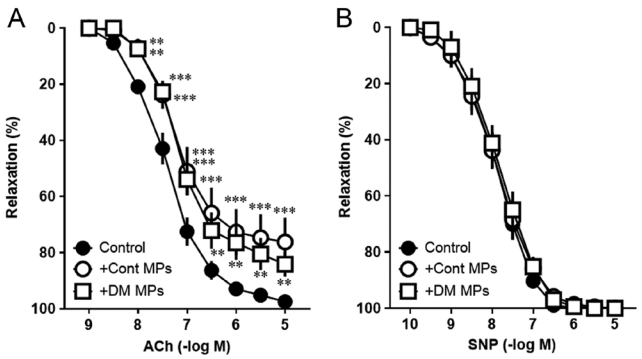

Figure 3
Effect of MPs on endothelium-dependent (A) or -independent (B) responses. Relaxation induced by ACh (A) and SNP (B) is shown in rings incubated for 30 min in the control, control MPs and DM MPs. The control MPs and DM MPs concentrations corresponded to plasma condition levels. Results are expressed as the mean ± S.E.; $n = 5$. $^{**}P < 0.01$, $^{***}P < 0.001$ vs Control.

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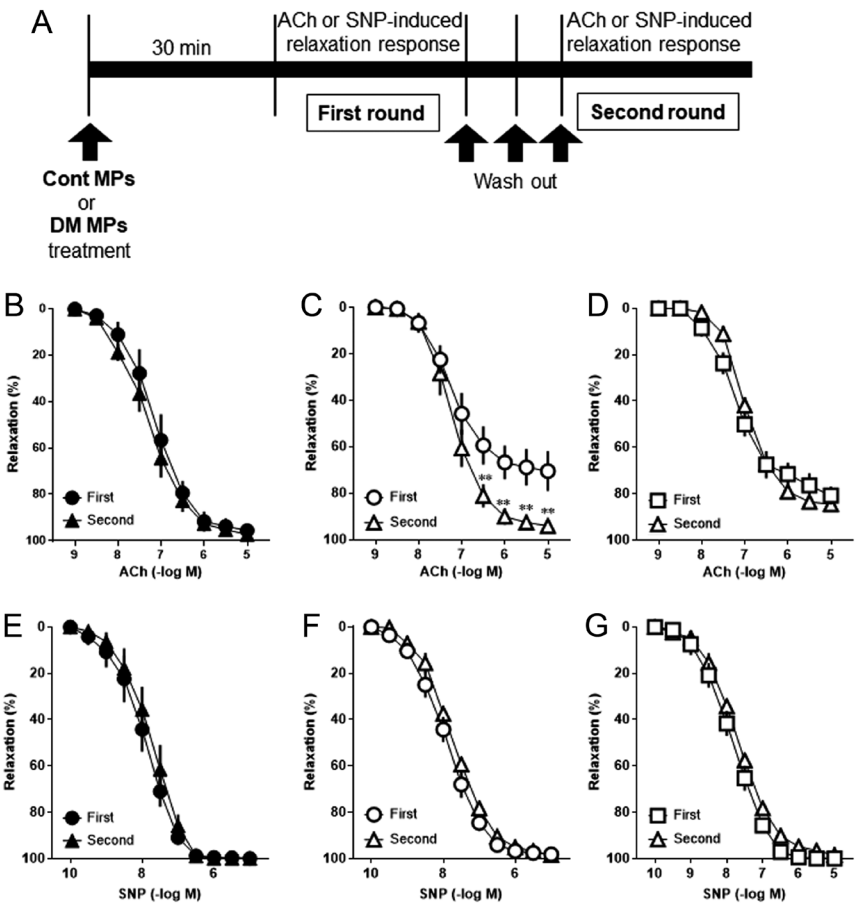

Figure 4
Effect of washed MPs on vascular relaxation. (A) Experimental protocol for aorta: control aortas were treated with vehicle, control MPs or DM MPs for 30 min as indicated. Thirty minutes after treatment, ACh or SNP-induced relaxation responses were measured. Subsequently, the aortas were washed out every 15 min, and again ACh or SNP-induced relaxation responses were measured. We named the first relaxation responses as the 'first round' and the next relaxation responses after washing out as the 'Second round'. (B, C, D, E, F and G) Relaxation induced by ACh (B, C and D) and SNP (E, F and G) is shown. (B and E) Vehicle treatment, (C and F) Control MPs treatment and (D and G) DM MPs treatment. Results are expressed as the mean ± S.E.; $n = 5$. $^{**}P < 0.01$ vs first round.

MP characterization

We performed an experiment to determine if MPs adhered to the endothelial cells of mice aorta. As shown in Fig. 5, CD42b-labeled Control and DM MPs were confirmed at the aorta surface (the endothelial cells) under the non-washed condition, but these Control MPs were not confirmed after washing. Interestingly, the labeling was mostly observed in the DM MP-treated aorta surface after washing.

To assess the contained protein of MPs, dot blot technique was employed notably to analyze the NO production pathway and MAPK. The mechanism of eNOS activation appears to be linked to an Akt pathway and MAPK family (ERK1/2, p38 MAPK and JNK) (Liu & Rockey 2013, Taguchi *et al.* 2016, 2017*a*,*b*). In this study, no signal was observed for Akt and p38 MAPK in Control MPs and DM MPs (Fig. 6A). Stronger signals for ERK1/2 and eNOS were observed in DM MPs than in Control MPs (Fig. 6A, B and C). Comparable signals for JNK and caveoloin-1 (a plasma membrane marker) were detected in DM MPs and Control MPs (Fig. 6A and D).

DM MPs regulated the activity and stability of eNOS

Endothelial cell membrane proteins have a very well-known pivotal role in multiple signaling events (Parton & Simons 2007). One of the highly studied signaling pathways related to endothelial cells is generation of NO through eNOS (Mineo & Shaul 2012). To elucidate the functional role of MPs in aorta, we measured NO under ACh stimulation and checked for the expression and activation of eNOS. As shown in Fig. 7A, NO levels were higher in the aortas treated with vehicle, Control MPs and DM MPs under ACh stimulation than under non-stimulation. However, NO levels in the mouse aortas treated with DM MPs under ACh-stimulation were significantly lower than those treated with Control MPs, although NO levels in all groups were similar under non-stimulation.

Phosphorylation of eNOS at serine 1177 activates NO production. So, we assessed the activated form of eNOS (p-eNOS) under ACh stimulation. There was no significant difference in p-eNOS expression among all groups (Fig. 7B and C), which suggested similar eNOS activation in aortas

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treated with DM MPs and Control MPs. Interestingly, we found that the total protein levels of eNOS were decreased in the aortas treated with DM MPs (Fig. 7A and D), although the total eNOS protein level was increased in aortas from DM mice compared with the aorta treated with DM MPs.

We hypothesized that MPs induced the release of new MPs, notably new MPs containing many eNOS proteins in DM. To test this, we measured eNOS levels in plasma by using an ELISA assay. As shown in Fig. 7E, the total eNOS protein levels in the plasma of DM mice were significantly increased relative to those in control mice. Furthermore, the total eNOS protein levels were significantly higher in DM MPs than in Control MPs (Fig. 7F). No differences in the p-eNOS protein levels and the inactivation of eNOS protein were observed between the Control MPs and DM MPs (Fig. 7G).

DM MPs were enhanced by ERK1/2 activation

We next investigated whether DM MPs could control the mechanisms of eNOS activation. The activation of eNOS by Akt and MAPK with a subsequent increase in NO production was an important downstream effector in endothelial cells (Liu & Rockey 2013, Taguchi *et al.* 2016, 2017*a*,*b*). Neither Control MPs nor DM MPs affected the expression of total and phosphorylated Akt and p38 MAPK in the aortas (Fig. 8A, B and C). Additionally, the expression and activation of Akt and p38 MAPK in the aortas treated with DM MPs and Control MPs were the same as those in DM mice. We thought that MPs did not have an effect on these molecules because these molecules were not contained in MPs. Furthermore, DM MPs increased the expression of total and phosphorylated ERK1/2 in the aortas when compared with Control MPs, and the p-ERK1/2 in the aortas treated with DM MPs were similar to those in the aortas from DM mice (Fig. 8A and D). JNK was also present in both Control MPs and DM MPs, but its effect on the expressions of total and phosphorylated JNK in the aortas was minimal (Fig. 8A and E).

Discussion

In this study, we demonstrated that circulating MPs from DM mice promoted the signaling pathway that caused the endothelial dysfunction. Furthermore, such DM MPs were exposed to control aortas. We found new results showing that ERK1/2-containing DM MPs led to the activation of ERK1/2 in the aortas, which led to the endothelial dysfunction in DM. Interestingly, we found that DM MPs adhered to the aortas and induced endothelial dysfunction, which was identified as the explanation for how DM MPs increased ERK1/2 levels and the ERK1/2 activity in aortas. Furthermore, DM MPs decreased eNOS levels and allowed release of eNOS. Interestingly, eNOS and ERK1/2 were contained within MPs. MPs are produced

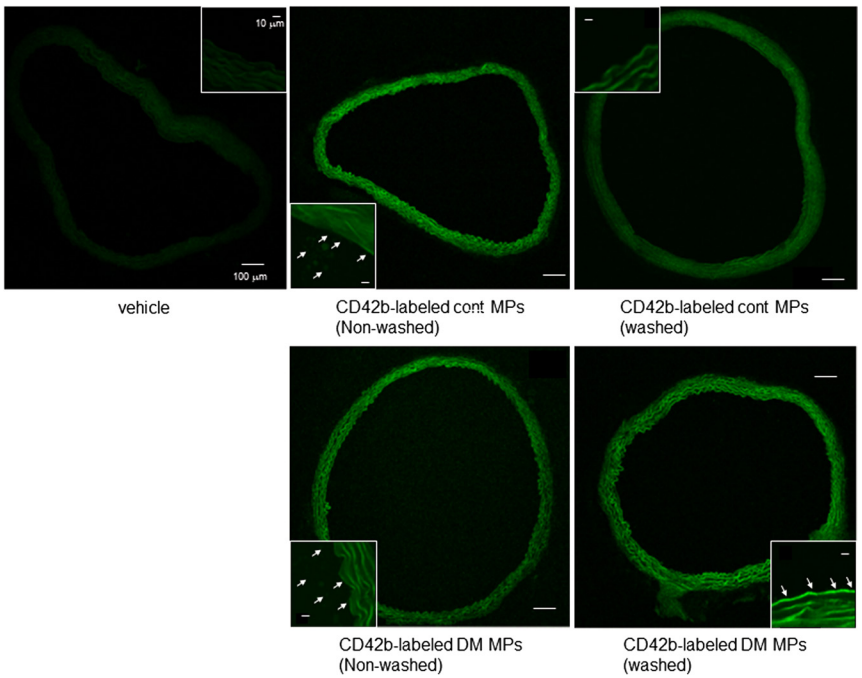

Figure 5
MPs adhered to aortas. Representative images of immunofluorescence staining show increased CD42b expression in aortas from control mice. Control or DM MPs were labeled with a DyLight 488-conjugated anti-mouse CD42b antibody, washed to remove any unbound antibody, and incubated with control aorta for 30 min (10,000 MPs/μL). Intense staining on the surface of aorta incubated with Control MPs can be observed but not after washing. However, the surface of aorta incubated with DM MPs after washing out reveals intense staining. These images are representative of five experiments. Scale bar = 100 μm. Scale bar in the small frame = 10 μm.

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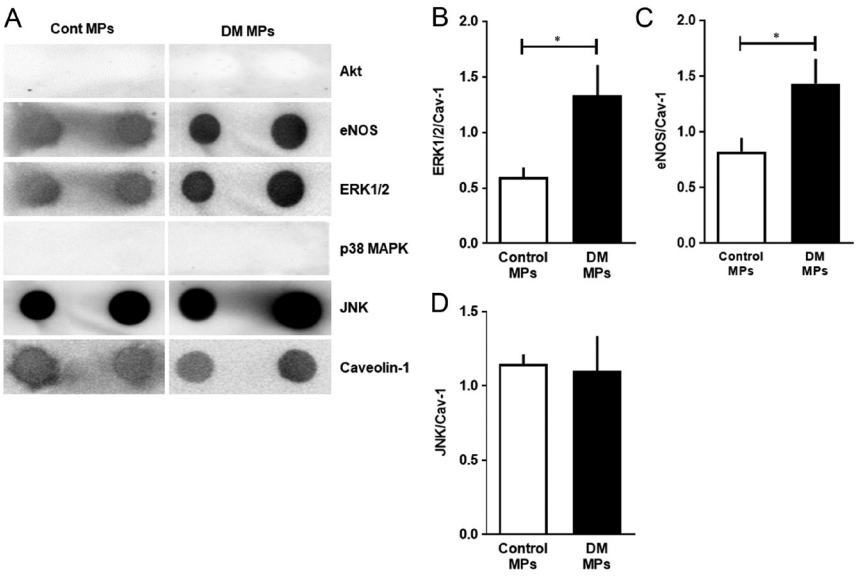

Figure 6
Characterization of MPs by dot blot assay. (A) Dot blot analysis of MPs of control and DM mice. Control MPs and DM MPs dot blotted and incubated with anti-Akt, anti-eNOS, anti-ERK1/2, anti-p38 MAPK, anti-JNK and anti-Caveolin-1 antibodies. Basically, Control MPs and DM MPs were run on a separated PVDF membrane under the same conditions. (B, C and D) Results of ERK1/2 (B), eNOS (C) and JNK (D) normalized to Caveolin-1. Results are expressed as the mean ± S.E.; $n = 6$. *$P < 0.05$ vs Control MPs.

and emitted by many types of cells, such as platelets, leukocytes and endothelial cells (Leroyer *et al.* 2007, Shantsila *et al.* 2010, Rautou *et al.* 2011). We considered that the ERK1/2-containing MPs and the eNOS-containing MPs were separately present from each other. ERK1/2 exists in various cell types, such as platelets and vascular cells. We cannot completely deny that ERK1/2-containing MPs come from endothelial cells. However, at least in this study, PDMPs increased in DM, and the treatment of ERK1/2-containing MPs increased total ERK1/2 levels in aortas. Additionally, eNOS-containing MPs come from endothelial cells because eNOS is a protein available only in endothelial cells. The production and emission of MPs occur via membrane abscission mechanism that is regulated by ERK1/2 (Muralidharan-Chari *et al.* 2009). So, it is considered reasonable and proper that ERK1/2-containing PDMPs adhered to the endothelial cells of aortas in DM. It is difficult to measure the ERK1/2 and eNOS protein in the MPs separated by labeled anti-CD42b, but we showed the adhesion between DM MPs and aortas using an immunostaining technique. Thus, our studies suggest the possibility that MP components regulate endothelial function and new MP release (Fig. 9). Furthermore, ERK1/2-containing DM MPs may contribute to the progression and development of diabetic vascular complications.

In the present study, we assessed the levels of PDMPs by measuring the number of CD42b-positive MPs by using flow cytometry and by using an ELISA kit. CD42b is a two-chain membrane glycoprotein found only on platelets and megakaryocytes (Fox *et al.* 1988). So, CD42b expression has been used as a marker of PDMPs (Piccin *et al.* 2007). Our results showed that circulating MPs are released from platelets into the extracellular space. Elevated levels of PDMPs have been associated with acute cardiovascular diseases, DM and hypertension (Nomura *et al.* 2003, Preston *et al.* 2003). The general consensus is that most cell types, such as platelets and endothelial cells, are capable of vesiculation and release membrane-shed MPs, especially under pathological conditions. Increases in intracellular calcium and oxidative stress promote MP release (Rautou *et al.* 2011). Additionally, pathological conditions lead MP formation (phosphatidylserine exposure on the cell membrane outer leaflet) and release (Rautou *et al.* 2011). Thus, calcium is regarded as an intrinsic pathogenic factor in many vascular diseases and has been observed to contribute to diabetic endothelial dysfunction. As shown in Fig. 2, PDMP levels increased in DM. However, it is not clear what is included in MPs. We proposed a hypothesis that the relative changes in the contents of MPs may reflect changes in adhesion to endothelium, which leads to decreased NO production and impaired vascular relaxation response.

The stimulus that triggers the production of MPs regulates the composition of these vesicles and, thus, the transfer of biological information (Martinez *et al.* 2011). Depending on the original cell type, cell surface composition and concentration, MP composition is a reflection of the original cell's redistributed plasma membrane (Tual-Chalot *et al.* 2010, Martinez & Andriantsitohaina 2011). MPs contain membrane, cytoplasmic and nuclear constituents specific to their cell of origin (Martinez *et al.* 2011). For example, proteins from PDMPs are surface glycoproteins or chemokines (Garcia *et al.* 2005). Furthermore, the lipid

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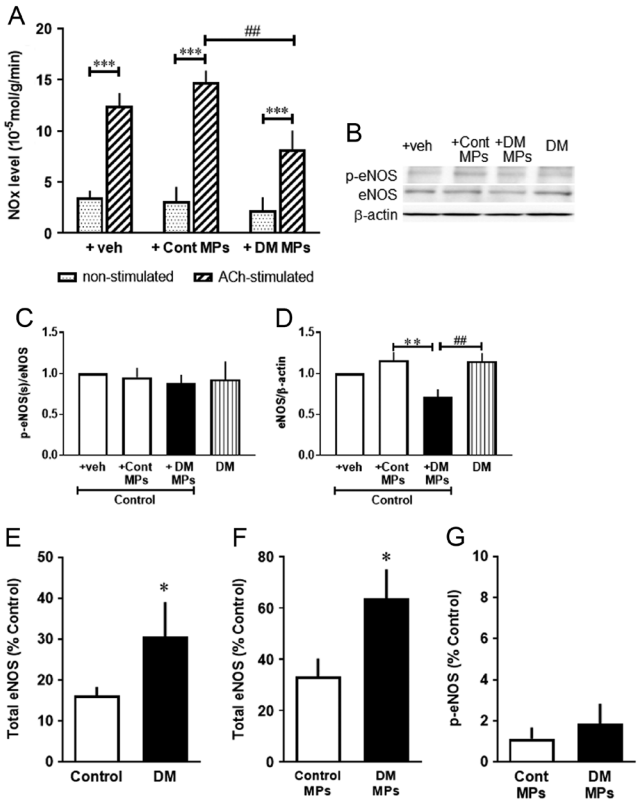

Figure 7

Effects of MPs on NO-related parameters. (A) Non or ACh-stimulated NO release from *ex vivo* aorta was measured by examining the production of nitrate and nitrite by using a NOx analyzer. Control aortas were treated with vehicle, Control MPs, or DM MPs for 30 min as indicated. Thirty minutes after treatment, ACh (10^{-6} mol/L) was stimulated for 20 min. Results are expressed as the mean ± S.E.; $n = 4–6$. ***$P < 0.001$ vs non-stimulated. ##$P < 0.01$ vs DM MPs. (B) Representative data of Western blot analysis of p-eNOS and eNOS in control aortas treated with vehicle, Control MPs, and DM MPs (30 min) and DM aortas under ACh-stimulation (20 min, 10^{-6} mol/L). Basically, control aortas treated with vehicle, Control MPs and DM MPs, and DM aorta were run on the same gel. (C) eNOS activity estimated by the eNOS phosphorylation at Ser1177. Densitometric analysis of density of bands for p-eNOS. The results were normalized to total eNOS protein. (D) Densitometric analysis of density of bands for eNOS. These results were normalized to β-actin. **$P < 0.01$ vs Control MPs. ##$P < 0.01$ vs DM MPs. (E, F and G) Plasma levels of total eNOS (E), total eNOS (F) and eNOS phosphorylation (G) in Control MPs and DM MPs, as measured by ELISA and expressed as the percentage increase vs. Control. Results are expressed as the mean ± S.E.; $n = 5–6$. *$P < 0.05$ vs Control or Control MPs.

environment can modify the activity of proteins carried by MPs. MPs also contain nucleic acids allowing for transfer of genetic material to target cells (Martinez *et al.* 2011). MPs consequently directly interact with ligands on target cells, activate cascade signaling and transfer proteins, mRNA and bioactive lipids (Martinez *et al.* 2011). However, inclusion of MPs is more poorly defined, notably in DM. In this study, we focused on the connection of DM MPs and endothelial dysfunction. So, we investigated the proteins that had been reported to be connected with endothelial dysfunction (Muniyappa & Sowers 2013, Ishida *et al.* 2014, Taguchi *et al.* 2016, 2017*a*, 2018). In this study, we suggested an absence of Akt and p38 MAPK in MPs and the presence of eNOS, ERK1/2, JNK and caveolin-1 in MPs. Notably, we showed that DM MPs expressed increased eNOS and ERK1/2. ERK1/2-containing MPs may be associated with ERK1/2 in aorta, itself absorbed to aorta because ERK1/2 increased with aorta. Furthermore, it is possible that some DM MPs could be derived from endothelial cells under the stimulation of PDMPs from DM because eNOS has been identified on endothelial MPs (Dignat-George & Boulanger 2011). This possibility corresponded with our results which eNOS in plasma and MPs from DM significantly increased. Further work is required to determine the exact origin of the MP populations and compositions and determine the mechanisms by which they are released from blood cells and/or platelets or from endothelial cells. However, DM MPs could be responsible at least in part for endothelial dysfunction in DM.

Endothelial-derived NO is the major mediator of ACh-induced vascular relaxation of aorta (Knight & Burnstock 1996). MPs from patients with myocardial infraction resulted in impaired ACh-induced relaxation and NO production (Boulanger *et al.* 2001). The same effect was seen by using circulating MPs obtained from DM rat (Ishida *et al.* 2016), endothelial-derived MPs obtained from HUVECs under quasi-diabetic condition (Taguchi *et al.* 2017*a*,*b*), and DM MPs in this study. This response has been shown to be abolished by a NOS inhibitor (Boulanger *et al.* 2001), which suggested that DM MPs-induced the vascular endothelial dysfunction via impairing the release of NO. Meanwhile, as shown in Fig. 3, Control MPs also impaired the endothelial relaxation response in aorta. The detailed mechanism is unknown. In this context, further research is required to be better understand how Control MPs control endothelial function, although we are of the opinion that Control MPs will reversibly link to the endothelial cells of aorta and primarily inactivate NO production signaling. Furthermore, as shown in Figs 3 and 4, we have data on twice-continuous ACh-induced relaxation responses in the presence of the suspended MPs (first round) and in their absence by washing out (second round). In aortic rings, the presence of the suspended MPs decreased ACh-induced relaxation. The washing out of the suspended DM MPs did not affect the decreased ACh-induced relaxation responses, but washing out of Control MPs increased those responses. This suggested that, in aortic rings, the adhesion of DM MPs may lead

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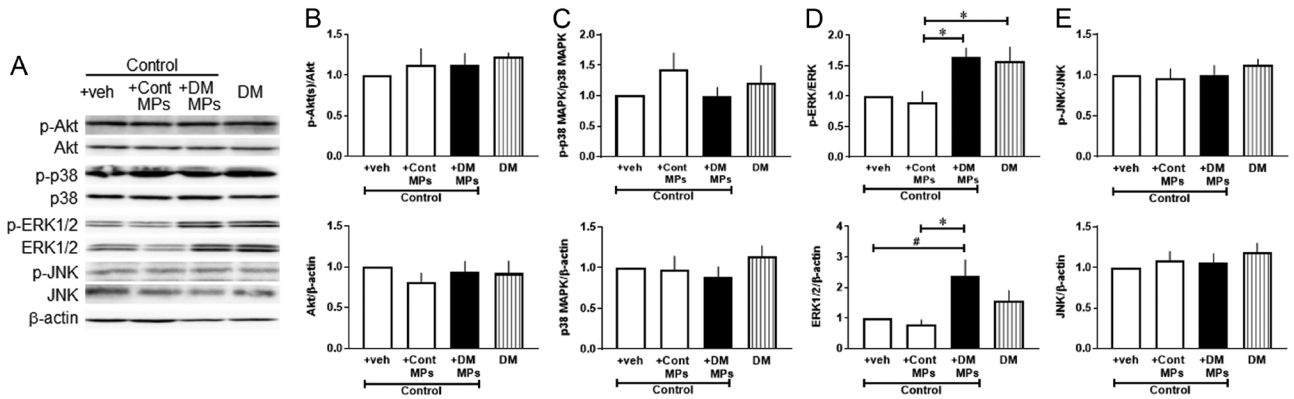

Figure 8

Effects of MPs on Akt and MAPK activity in aortas under non-stimulation. (A) Representative data of Western blot analysis of p-Akt (Ser 473), Akt, p-p38 MAPK (Thr180/Tyr182), p38 MAPK, p-ERK1/2 (Thr202/Tyr204), ERK1/2, p-JNK (Tyr185), and JNK. Basically, control aortas with vehicle, Control MPs and DM MPs, and DM aorta were run on the same gel. (B, C, D and E) Top, the phosphorylation levels of Akt (B), p38 MAPK (C), ERK1/2 (D), and JNK (E) in the control aortas treated with vehicle, Control MPs and DM MPs and DM aortas. Bar graph showing densitometric data for p-Akt, p-p38 MAPK, p-ERK1/2 and p-JNK against these total proteins. Bottom, total Akt (B), p38 MAPK (C), ERK1/2 (D) and JNK (E) in the control aortas treated with vehicle, Control MPs, DM MPs and DM aortas. Bar graph showing densitometric data for Akt, p38 MAPK, ERK1/2 and JNK against β-actin. Results are expressed as the mean ± S.E.; $n = 6$. $^{*}P < 0.05$ vs Control MPs. $^{\#}P < 0.05$ vs vehicle.

to diminished NO production which may, in turn, trigger an impairment of endothelial-dependent relaxation. The results of Fig. 7A show NO production under the absence of the suspended MPs after washing the aortas. So, ACh-stimulated NO production was less in the treatment of DM MPs compared with Control MPs.

As shown in Fig. 4, we indicated the involvement of DM MPs in the mechanism leading to the diabetes-induced endothelial dysfunction, and the difference in characteristics between Control MPs and DM MPs because the Control MPs did not adhere strongly to the aortas, whereas DM MPs adhered strongly to the aortas and could not be removed. Next, we analyzed eNOS expression and activity in aortas under circulating MPs stimulation, as circulating MPs from subjects with vascular dysfunction originate largely from the endothelium (Horstman *et al.* 2004). Our results indicated that the endothelial dysfunction induced by DM MPs was likely to be caused by a decrease in eNOS expression. However, the aortas with long-standing DM did not decrease eNOS protein at all. This finding was in agreement with that of our previous study (Taguchi *et al.* 2016). Although we can hypothesize that improved eNOS levels are a compensatory mechanism against reduced levels of eNOS protein in DM, further experiments are needed to investigate this hypothesis (for example, long duration exposure of DM MPs). Although the cellular mechanism of action of MPs remains to be investigated, our data demonstrated that DM MPs affected eNOS expression because stimulation of DM MPs results in a decrease in aortic eNOS protein expression and an increase in blood eNOS levels, which causes DM MPs-derived endothelial MPs to be released into the bloodstream and

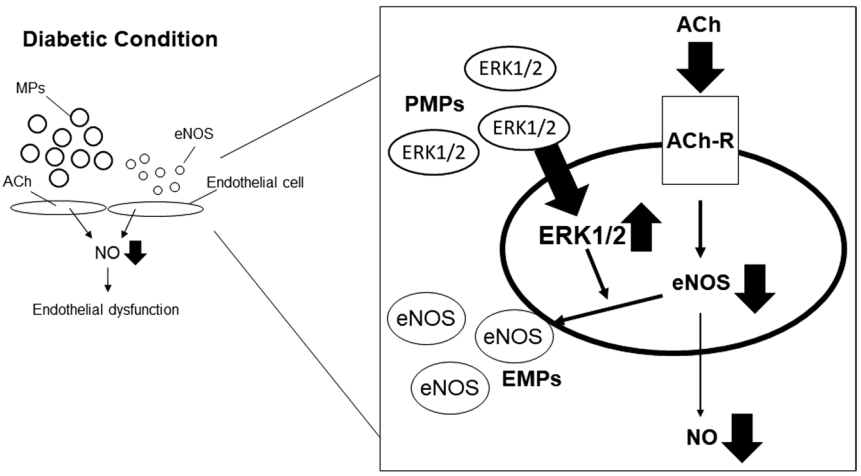

Figure 9

Schematic diagram demonstrating the molecular mechanisms by which DM MPs induce endothelial dysfunction. ACh-R, ACh-receptor (muscarinic receptor); EMPs, endothelium-derived MPs; PMPs, platelet-derived MPs.

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impair endothelial-dependent relaxation in response to ACh on aortas in mice. Furthermore, our results indicated that the effects induced by DM MPs were not only independent of the number of MPs but also dependent on the different compositions between control and DM MPs. In addition, an effect of MPs on the mechanism of action of NO on vascular smooth muscle can be ruled out because the relaxation to SNP was unaltered.

The Akt signal pathway is important for regulation of eNOS expression and/or activity (Taguchi *et al.* 2016, 2017*a*,*b*). However, in the present study, MPs from either Control or DM did not produce the changes in upregulation of Akt expression and phosphorylation in aortas. These results indicated that non-Akt-containing MPs modified the eNOS pathway regardless of the aortic Akt signal pathway. We then evaluated the role of MAPK under MPs stimulation. It is well known that MAPKs, such as p38 MAPK and ERK1/2, increased in response to hyperglycemia and were involved in cardiovascular complication in diabetes (Westermann *et al.* 2006, Rajesh *et al.* 2010). In the present study, we clearly showed augmented p-ERK1/2 and total ERK1/2 in aortas treated with DM MPs and from DM mice, which suggested the involvement of the ERK1/2 pathway in the DM MPs-induced endothelial dysfunction. ERK1/2 occurs in both endothelial cells and smooth muscle cells in the aorta. Probably, both endothelial cells and smooth muscle cells suffered under the influence of DM MPs. Consistent with these results, ERK1/2 has recently been shown to be involved in endothelial extracellular vesicles-induced endothelial dysfunction by regulating eNOS protein levels (Taguchi *et al.* 2017*b*). Here we think that ERK1/2 in endothelial cells has a suppressive role in NO production, although they affected the smooth muscle cells in different ways. Interestingly, ERK1/2 increased in DM MPs. Thus, the findings of the present study suggest the existence of cross-talk between the ERK1/2-containing DM MPs and aortic ERK1/2. Endothelial MP formation requires activation of the RhoA–ROCK pathway during thrombin and Ang II stimulation (Sapet *et al.* 2006, Burger *et al.* 2011). Furthermore, Vion *et al.* (2013) reported that ROCK and ERK1/2 increased endothelial MPs release, whereas the p38 MAPK and JNK pathways were without effect. Our data suggested that ERK1/2-containing DM MPs fused with endothelial membrane and ERK1/2 in the aortas stimulated release of eNOS-containing endothelial MPs, which led to reduced eNOS protein in the aortas and impaired NO production. To our knowledge, this is the first study to describe the relationship between DM MPs-mediated ERK1/2 activation and eNOS expression in aortas, although additional work is needed to confirm (1) the mechanisms of adhesion of ERK1/2-containing DM MPs to the endothelium and of integration of ERK1/2 with aortic ERK1/2 and (2) the pathway associated with release of endothelial MPs by aortic ERK1/2.

In summary, we demonstrated that diabetic mice produced PDMPs containing abundant ERK1/2, which decreased eNOS protein and NO production in aorta and led to DM MPs-mediated impairment of vascular relaxation. Our findings indicate that modulation of vascular function by DM MPs appears to be eNOS protein levels dependent and may involve ERK1/2. Furthermore, DM PDMPs may release endothelial MPs that contain abundant eNOS, and future studies should determine if ERK1/2-containing PDMPs contribute to this. However, cross-talk between MPs and endothelial cells may be important in diabetic conditions associated with vascular injury and increased endothelial MP formation. PDMPs containing ERK1/2 might provide a therapeutic target for diabetic vascular complications.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

K T and T K conceived, designed the study and wrote the manuscript. K T and H N performed experiments. K T and H N analyzed data. T M contributed to the study design and interpreted data. T K edited the manuscript.

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