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ARTICLE

Co-treatment with clonidine and a GRK2 inhibitor prevented rebound hypertension and endothelial dysfunction after withdrawal in diabetes

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Abstract

Hypertension and diabetes are associated with a risk of cardiovascular disease. Clonidine is currently used as a fourth-line drug therapy for hypertension because of its rebound hypertensive effect and short half-life. The purpose of this study was to investigate the combined effect of an antihypertensive drug (clonidine) and a G-protein-coupled receptor kinase 2 (GRK2) inhibitor on rebound hypertension and endothelial dysfunction. The clonidine and/or GRK2 inhibitor were administered by continuous infusion for 14 days by using an osmotic pump that was implanted subcutaneously. To test the effects of GRK2 inhibitotr, we measured blood pressure by using a tail-cuff system in diabetic mice in which rebound hypertension was induced by withdrawal after clonidine treatment and measured vascular responses in isolated aortas from these mice. The mice were then euthanized 7 days later. We observed that, in diabetes mellitus (DM) mice, blood pressure began to decline after 3 days of clonidine or clonidine + GRK2-inhibitor infusion. However, 15 days after initiation of treatment, the blood pressure of the clonidine only-treated DM mice began to increase and resulted in a high final blood pressure. At 21 days, clonidine withdrawal triggered rebound hypertension together with impaired endothelium-dependent relaxation, increased GRK2 activity, and reduced Akt/endothelial NO synthase (eNOS)/NO production in aortas. Conversely, withdrawal of the combination clonidine/GRK2-inhibitor treatment did not cause rebound hypertension, and normal induction of endothelium-dependent relaxation, decreased GRK2 activity, and increased Akt/eNOS were observed in aortas from DM mice. These results suggest that suppression of GRK2 activity affects rebound hypertension-associated vascular endothelial dysfunction by targeting the Akt/eNOS signaling pathway.

Introduction

Type 2 diabetes mellitus (DM), and hypertension often occur simultaneously [1, 2]. In addition, there is general agreement that coexistence of DM and hypertension is associated with high cardiovascular risk and mortality in humans and animal models [3–5]. The symptom of cardiovascular disease is endothelial dysfunction, which is related to poorer prognosis [6, 7]. A previous study defined endothelial dysfunction as an impaired endothelium-dependent relaxation response, which is a key measure of endothelial function [8]. The present study, therefore, provides additional and strong evidence in favor of a causal interaction between endothelial dysfunction, diabetes, hypertension, and the pathogenesis of cardiovascular events.

Vascular reactivity and blood pressure homeostasis are controlled via a complex network of cell signaling mechanisms [9]. Among the network of receptors and signaling molecules regulating blood vessel reactivity, G-protein-coupled receptors (GPCRs) [10], such as adrenergic receptor and angiotensin II (Ang II) receptor are known to have a central role and represent an important target for treatment of hypertension [9]. Of particular importance, GPCRs are phosphorylated by a family of G-protein-coupled receptor kinases (GRKs) [10]. Among the seven GRK isoforms, GRK2 is the most abundant in blood vessels and has a fundamental role in control of systemic vascular responses [10, 11]. One function of

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G-proteins is to activate GRK2 that in turn phosphorylate the specific receptor for desensitization, which leads to recruitment of β-arrestin 2. Subsequently, β-arrestin 2 binds to the GRK2-phosphorylated motifs of the receptor and induces receptor internalization. This GPCR desensitization/internalization is agonist specific and GRK2 dependent. The levels and activity of the GRK2 isoform were increased in animal models of hypertension and diabetic vascular complications [12–14]. Furthermore, the interaction of GRK2 with Akt has been shown to inhibit Akt-dependent activation of endothelial nitric oxide (NO) synthase (eNOS), thus impairing NO production and vascular reactivity, which leads to endothelial dysfunction [14, 15]. We and others have reported that GRK2 inhibition was vasoprotective and antihypertensive in various animal models [11, 16–20].

Many antihypertensive drugs have been developed and proven to be effective in controlling blood pressure. Myriad pharmacological agents exist for the treatment of hypertension, including β-blockers, diuretics, angiotensin-converting enzyme inhibitors, calcium channel blockers, and Ang II-receptor blockers (ARBs). However, many patients treated for hypertension do not achieve their target blood pressure. There are several reasons for poor blood pressure control, including poor patient compliance with treatment. In addition, the risk of acute withdrawal and rebound hypertension is well recognized with sudden cessation of systemically administered partial antihypertensive drugs [21]. In this study, we describe a case of rebound hypertension after clonidine withdrawal in DM mice. Clonidine is classified as an alpha-2 adrenergic receptor agonist and has been used for several years to treat hypertension. It is currently considered a fourth-line drug treatment because of side-effects, such as rebound hypertension. However, clonidine induces NO production and the endothelium-dependent relaxation response via Akt/eNOS signaling [22]. Furthermore, we found that in mouse aortas, the clonidine-induced relaxation response was regulated by GRK2 activity [13]. We further noted that losartan, a specific ARB, improved endothelial dysfunction by reducing GRK2 activity [13], but there was no evidence of rebound hypertension after losartan withdrawal [23]. Taken together, it appears that suppression of GRK2 activity reduces rebound hypertension. However, the detailed molecular mechanisms and the relevant functional interactions underlying the deleterious effects of clonidine withdrawal-induced rebound hypertension and the beneficial effects of GRK2 inhibition have not yet been fully demonstrated.

We had previously focused on Ang II-associated endothelial dysfunction via the GRK2 pathway by using an osmotic pump [13]. However, examination of the effects related to cessation of pump infusion resulting in rebound hypertension after withdrawal of a drug has not been reported.

Therefore, the purposes of this study were (1) to determine potential changes in blood pressure after expiration of drug (clonidine and/or a GRK2 inhibitor) infusion in diabetic mice and (2) to examine the role of GRK2 and endothelial dysfunction/GRK2 interactions in rebound hypertension. To determine potential changes in blood pressure and vascular reactivity, osmotic pumps were used in DM mice. These pumps were used to administer clonidine and/or a GRK2 inhibitor or vehicle. The doses of the drugs were based on previous reports [13, 24].

Methods

Animals

This study was approved by the issuing committee (Committee on the Care and Use of Laboratory Animals of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan). Four-week-old male Institute of Cancer Research (ICR) mice were supplied by Tokyo Animal Laboratories. All experiments were performed under the institutional guidelines for the humane treatment of laboratory animals. To induce DM, 5-week-old male mice received an intraperitoneal injection of 1.5 g kg^{-1} nicotinamide (Nic; Sigma-Aldrich, St. Louis, MO, USA) dissolved in saline 15 min before an injection via the tail vein of streptozotocin (STZ; 200 mg kg^{-1}, Sigma-Aldrich) dissolved in a citrate buffer [13, 19, 22]. Age-matched control mice received saline followed by citrate buffer (via the routes mentioned above). Ten weeks after this treatment, for chronic drug administration, vehicle, clonidine (125 μg kg^{-1} day^{-1}, Sigma-Aldrich), and/or a GRK2 inhibitor (methyl[5-nitro-2-furyl] vinyl)-2-furoate; 5.64 ng kg^{-1} day^{-1}; Calbiochem, La Jolla, CA, USA) were infused subcutaneously into DM mice by using osmotic pumps (mode 1002, ALZET, Cupertino, CA, USA) for 2 weeks. The animals were then left to rest for 7 days before experimentation.

Blood pressure measurement

Blood pressure measurements were performed every 2 or 3 days. BP98A (Softron, Tokyo, Japan) was used to measure blood pressure by using the tail-cuff method, as described previously [13, 19, 22, 25, 26]. The small animal study unit of the system has a mouse holder base with a built-in warming element to raise the ambient temperature to 37 °C and maintain adequate circulation in the tail for indirect systolic blood pressure (SBP), mean blood pressure (MBP), diastolic blood pressure (DBP), and heart rate measurements. Because normal blood pressure shows intrinsic diurnal variation and may be disturbed by

environmental conditions, all measurements were performed in a quiet room during the morning. Before the start of the experimental protocol, all mice had been habituated to the blood pressure recording procedure.

Measurement of body weight and various parameters

Body weight was measured and blood samples were collected from all five groups (control group, DM group, clonidine-treated DM group, GRK2-inhibitor-treated DM group, clonidine + GRK2-inhibitor-treated DM group) 21 days after osmotic pump implantation. After mouse euthanasia, blood was collected and stored in tubes containing EDTA (Sigma-Aldrich) on ice. Blood samples were centrifuged at 3000 × *g* for 10 min at 4 °C, and plasma was extracted immediately. Plasma glucose levels were measured by using a commercial kit (Wako Chemical Company, Osaka, Japan), and plasma insulin concentrations were determined by using a competitive ELISA (Shibayagi, Gunma, Japan). Ang II levels were assessed by using an enzyme immunoassay kit (SPI-Bio, Massy, France) in accordance with the manufacturer's instructions.

Analysis of protein expression by western blotting

Aortic rings were frozen in liquid nitrogen and homogenized in ice-cold RIPA lysis buffer (Thermo Scientific, Rockford, IN, USA) containing a protease and phosphatase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). The lysates were centrifuged at 13,000 × *g* for 10 min at 4 °C, as previously described [13, 14, 19, 22, 25–29]. The supernatants were collected and the protein concentration was determined by using a BCA protein assay kit (Pierce, Rockford, IL, USA). Samples containing 25 μg of protein were loaded into lanes of 10% sodium dodecyl sulfate polyacrylamide gel and subjected to electrophoresis. The resolved proteins were transferred to a polyvinyl difluoride membrane and blocked with ImmunoBlock (DS Pharma Biomedical, Suita, Japan). Primary antibodies against GRK2 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated GRK2 at Ser670 (1:1000, Gene Tex Inc., Irvine, CA, USA), Akt (1:1000, Cell Signaling Technology, Danvers, MA, USA), phosphorylated Akt at Ser473 (1:1000, Cell Signaling Technology), eNOS (1:1000, BD Biosciences, San Jose, CA, USA), phosphorylated eNOS at Ser1177 (1:1000, Cell Signaling Technology), and β-actin (1:5000, Sigma-Aldrich) were used for incubation at 4 °C overnight. This was followed by incubation with horseradish peroxidase-conjugated IgG (1:10,000, Promega, Madison, WI, USA) for 20 min at 37 °C. The blots were detected by using a SuperSignal (Thermo Scientific). To normalize the data, we used β-actin as a housekeeping protein. Ratios of the optical densities of phosphorylated GRK2, Akt, or eNOS to the optical densities of the corresponding total protein bands were calculated. GRK2 activity was taken to be the reciprocal of the phosphorylated GRK2 expression.

Vascular relaxation of the aorta

Vascular reactivity of the mouse aorta was assessed as previously described [13, 14, 19, 22, 25, 26]. The thoracic aorta was removed and dissected free from adherent fat and connective tissue before being cut into ring segments of 2 mm width. Vascular rings were suspended in 10-ml organ baths containing Krebs–Henseleit solution (KHS) that was maintained at 37 °C and aerated with a 5% CO_2/95% O_2 mixture. The KHS composition was as follows: 118.0 mM NaCl, 4.7 mM KCl, 25.0 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgSO_4$, and 11.0 mM glucose. Each ring segment was attached to a force displacement transducer, as previously described. A resting tension of 1.5 g was applied to each segment.

After an equilibration period of at least 45 min, KCl (80 mM) was added to the bath, and the contractility of the segment was examined. Next, the KHS was replaced every 15 min. The cumulative concentration–response curves for acetylcholine (ACh; 10^{-9}–10^{-5} M), sodium nitroprusside (SNP; 10^{-10}–10^{-5} M), clonidine (10^{-9}–10^{-6} M) or insulin (10^{-9}–3×10^{-6} M) were obtained at the stabilized peak of the contraction (1 g tension state) evoked by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-6}–3×10^{-6} M). The results are expressed as a percentage of the $PGF_{2\alpha}$-induced contraction.

Measurement of NOx levels

NOx levels were determined by an NO detector/high-performance liquid chromatography system (ENO-20; Eicom, Kyoto, Japan) as previously described [13, 14, 22]. Briefly, each aorta was incubated in KHS with or without ACh (10^{-6} M) at 37 °C for 20 min. After incubation, the effluent was collected and analyzed, which allowed NOx levels to be calculated and used as an indicator of total NO production. Finally, the aortas were used for determination of protein expression by western blotting.

Statistical analysis

Statistical analysis was performed by using GraphPad Prism (version 6, GraphPad Software). Values are expressed as the mean ± standard error of the mean (SE). Vascular reactivity was analyzed by using two-way repeated measures ANOVA followed by a post hoc Bonferroni test. Western blot and NOx data were analyzed by Tukey's test followed

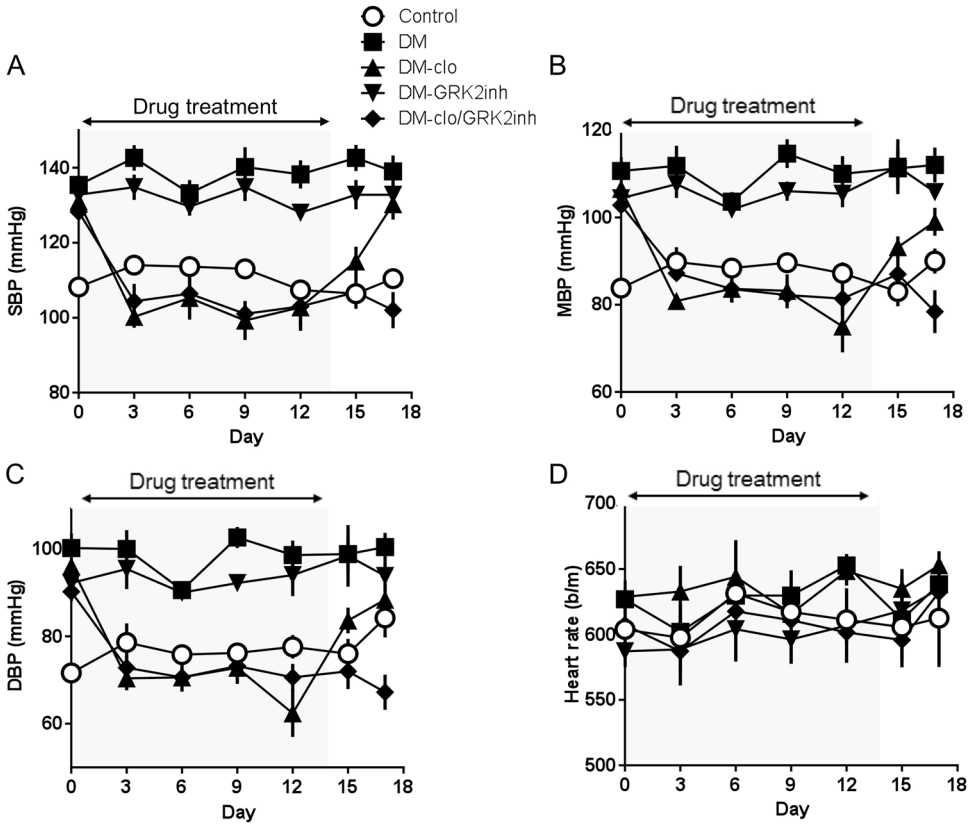

Fig. 1 Effects of drug withdrawal on blood pressure and heart rate in DM mice. **a** Systolic blood pressure (SBP), **b** mean blood pressure (MBP), **c** diastolic blood pressure (DBP), and **d** heart rate. SBP, MBP, DBP, and heart rate before, during, and after systemic infusion of clonidine and/or the GRK2 inhibitor in control or DM mice were determined every 2 or 3 days. Control, control mice that were sham-operated; DM, DM mice that were sham-operated; DM-clo, DM mice that received clonidine infusions (125 μg kg^{-1} day^{-1}) via an osmotic pump for 2 weeks and were then left to rest; DM-GRK2inh, DM mice that received GRK2-inhibitor infusions (5.64 ng kg^{-1} day^{-1}) via an osmotic pump for 2 weeks and were then left to rest; DM-clo/GRK2inh, DM mice that received infusions of clonidine and the GRK2 inhibitor via an osmotic pump for 2 weeks and were then left to rest. Values represent the mean ± SE; $n = 5$

by a multiple comparison test. P values of <0.05 were considered as indicating statistical significance.

Materials

Clonidine chloride, Nic, and STZ were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The GRK2 inhibitor (methyl [(5-nitro-2-furyl) vinyl]-2-furoate) was purchased from Calbiochem (San Diego, CA, USA). $PGF_{2\alpha}$ was purchased from Fuji Pharma Co., Ltd. (Tokyo, Japan); ACh chloride was purchased from Daiichi-Sankyo Pharmaceuticals (Tokyo, Japan); and SNP was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The concentrations of these drugs are expressed as final molar concentrations. Antibodies for Akt, Akt phosphorylated at Ser473 and eNOS phosphorylated at Ser1177 were purchased from Cell Signaling Technology (Danvers, MA, USA); the antibody for eNOS was purchased from BD Biosciences (San Jose, CA, USA); the antibody for GRK2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); the antibody for GRK2 phosphorylated at Ser670 was purchased from GeneTex Inc. (Irvine, CA, USA); the antibody for β-actin was purchased from Sigma Chemical Co. Horseradish peroxidase-linked secondary anti-mouse and anti-rabbit antibodies were purchased from Promega (Madison, WI, USA). All antibodies were diluted to appropriate concentrations.

Results

Effect of the GRK2 inhibitor on rebound hypertension in DM mice

Although blood pressure levels were higher in sham-operated DM mice than to those in sham-operated control mice, only infusion of the GRK2 inhibitor alone had no effect on blood pressure levels during the experimental period in the DM mice (Fig. 1). When DM mice were implanted with osmotic pumps that delivered 125 μg kg^{-1} day^{-1} of clonidine with or without 5.64 ng kg^{-1} day^{-1} of the GRK2 inhibitor, they showed a significant decrease in

Table 1 Effects of drug withdrawal on blood pressure and heart rate 21 days after implantation of an osmotic pump in DM mice

Parameters	Control	DM	DM-clo	DM-GRK2inh	DM-clo/GRK2inh
SBP (mmHg)	108 ± 4	131 ± 4***	125 ± 1**	126 ± 2**	110 ± 4$^{\#\#\#\$}$
MBP (mmHg)	85 ± 3	106 ± 2***	100 ± 2*	106 ± 2***	88 ± 4$^{\#\#\$}$
DBP (mmHg)	73 ± 2	93 ± 3***	84 ± 3	99 ± 2***	78 ± 4$^{\#\#}$
Heart rate (beats per min)	595 ± 13	621 ± 12	665 ± 10	623 ± 25	635 ± 16

Values are means ± SE ($n = 5$)

SBP systolic blood pressure, *MBP* mean blood pressure, *DBP* diastolic blood pressure

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. Control, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. DM, $^{\$}P < 0.05$ vs. DM-clo.

blood pressure at 12 days. As the resting state at the end of the drug-infusion period continued, the decrease in blood pressure could not be sustained from 15 days in the DM mice that had received clonidine only. In the 15th day's data (1 day after clonidine withdrawal) the rapidly increased blood pressure decreased very slowly in the clonidine-treated DM mice. The half-time of clonidine is ~20 h. These observation and characteristics suggested that there was leakage of clonidine from the osmotic pump until the 14th day, and as of the 15th day, clonidine remained in the blood of the clonidine-treated DM mice. On the 17th day, clonidine withdrawal led to rapid increases in the DM mice's blood pressure, which suggested that clonidine withdrawal caused rebound hypertension. No group exhibited a change in heart rate over the 17 days of measurement.

To confirm a diagnosis of rebound hypertension, we measured blood pressure and heart rate at 21 days (7 days after the end of clonidine and/or GRK2-inhibitor infusion). The SBP, MBP, and DBP were higher in the DM mice, clonidine-treated DM mice, and GRK2-inhibitor-treated DM mice than in the control mice, whereas co-treatment with clonidine and the GRK2 inhibitor significantly attenuated the elevation of SBP, MBP, and DBP in the DM mice (Table 1). There were no significant differences in heart rates among the groups (Table 1). Therefore, it appeared that clonidine withdrawal induced rebound hypertension at 21 days after the implantation of an osmotic pump (7 days after the end of clonidine release). This time point (that is, day 21 after implantation) was subsequently selected for further evaluation.

General animal data

Throughout 2 weeks of continuous clonidine and/or GRK2-inhibitor infusion and 1 week of rest, all animals in this study were well and healthy and did not demonstrate any significant changes in behavior. Body weights generally remained stable or increased slightly. There were no significant differences in body weight among the groups during the study, and no adverse events related to treatment were identified.

Body weight did not differ among the groups (Fig. 2a). The DM mice had elevated plasma glucose levels relative to those of controls, which were unaffected by treatment with clonidine and/or the GRK2 inhibitor (Fig. 2b). As expected, plasma insulin levels did not differ significantly between the control and drug-treated DM mice (Fig. 2c), suggesting that the infusions of clonidine and/or the GRK2 inhibitor by the osmotic pumps did not affect DM. Interestingly, plasma Ang II levels were significantly higher in the DM mice and drug-treated DM mice than in the control mice (Fig. 2d), although the blood pressures (SBP, MBP, and DBP) of the clonidine/GRK2-inhibitor-cotreated DM mice were significantly lower than those of the DM mice (Table 1).

The GRK2 inhibitor induced sustained suppression of GRK2 activation

Increased GRK2 levels have been reported in patients with metabolic disorder and cardiovascular diseases [30, 31]. Furthermore, GRK2 downregulation has been shown to prevent development of metabolic disorders [13, 14, 19, 32]. In addition, as stated above, the GRK2 inhibitor had a sustained effect on blood pressure, even though infusions of the drug had stopped. Thus, we evaluated the possibility that the GRK2 inhibitor could regulate the activation of GRK2 signaling in DM mice. To investigate this, we first measured GRK2 levels and GRK2 activity. Aortic GRK2 protein levels were significantly higher in the DM mice and drug-treated DM mice than in the controls, but there were no differences in GRK2 levels between the DM mice and drug-treated DM mice (Fig. 3a). Consistently, GRK2-inhibitor-treated DM mice and clonidine/GRK2-inhibitor-cotreated DM mice showed reduced GRK2 activity, although the DM mice and clonidine-treated DM mice demonstrated increased activity (Fig. 3b).

Effects of the GRK2 inhibitor on endothelial function in rebound hypertension in DM mice

We determined whether clonidine and/or GRK2 inhibitor withdrawal induced or improved endothelial dysfunction

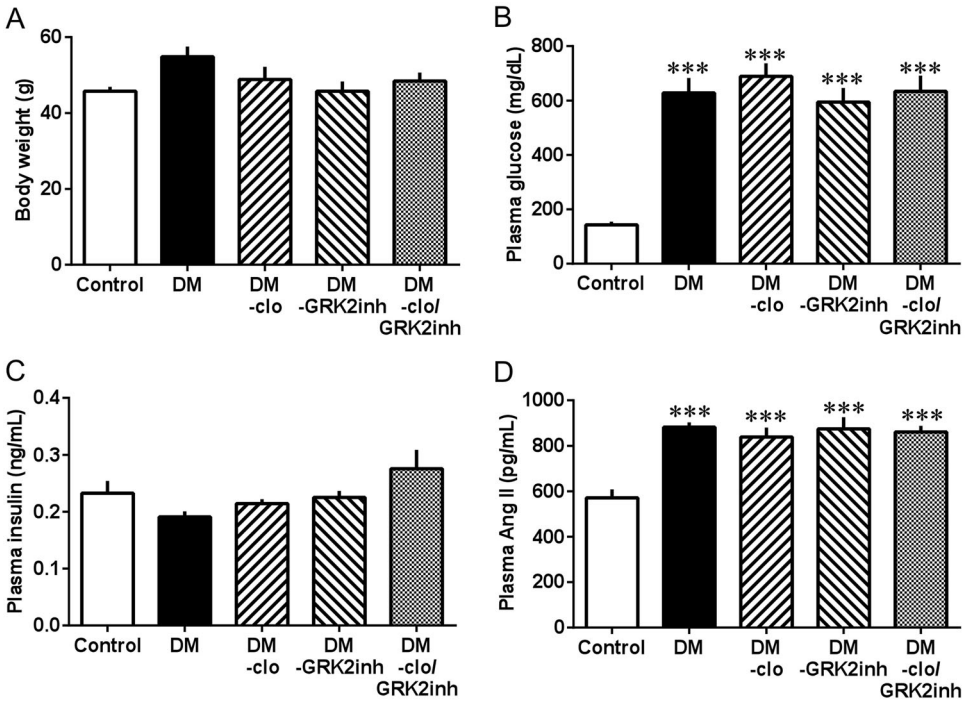

Fig. 2 Effects of drug withdrawal on various parameters 21 days after implantation of an osmotic pump in DM mice. **a** Body weight, **b** non-fasting plasma glucose levels, **c** non-fasting plasma insulin levels, and **d** plasma angiotensin II (Ang II) levels. Values represent the mean ± SE; $n = 5$. ***$P < 0.001$ vs. Control

in vivo by isometric tension measurement. Clonidine withdrawal significantly impaired ACh-induced endothelium-dependent relaxation of the isolated aortic rings of DM mice, which was restored by the GRK2 inhibitor (Fig. 4a). However, it did not affect endothelium-independent relaxation induced by the NO donor SNP (Fig. 4b). Furthermore, to evaluate whether a different route exerted a relaxant effect on GRK2-inhibitor-treated DM mice, we performed clonidine- and insulin-induced endothelium-dependent relaxation response curves. Clonidine- and insulin-induced relaxation was markedly reduced in the DM mice, GRK2-inhibitor-treated DM mice, and clonidine-treated DM mice (Fig. 4c, d). However, the clonidine and GRK2-inhibitor-cotreated DM mice showed the same response as that of the control mice. We found that the interaction between the GRK2 inhibitor and clonidine was localized to the vascular endothelium.

Effects of the GRK2 inhibitor on NO production stimulated by ACh in aortas from DM mice

One of the main functions of the endothelium is to produce NO, which is catalyzed by eNOS. We next studied whether clonidine and/or GRK2-inhibitor withdrawal affected ACh-stimulated NO production. As seen in Fig. 5a, there was decreased NO production in aortas from clonidine-treated DM mice, which was reversed in aortas from clonidine/GRK2-inhibitor-cotreated DM mice. Interestingly, clonidine/GRK2-inhibitor co-treatment in DM mice increased basal aortic NO levels relative to those observed in control mice (Fig. 5b).

To elucidate the molecular mechanisms leading to increased basal NO production, protein levels of eNOS and upstream Akt were determined. We observed a significant increase in p-eNOS (Ser1177)/eNOS in aortas from the clonidine/GRK2-inhibitor-cotreated DM mice relative to that of the controls or DM mice, whereas no differences in total aortic eNOS were observed between the groups (Fig. 5c). Furthermore, western blot analysis revealed that p-Akt was significantly increased only in aortas from the clonidine and GRK2-inhibitor-cotreated DM mice, whereas total Akt levels did not differ among the groups (Fig. 5d).

Discussion

In this study, we investigated the potential interactions among rebound hypertension, GRK2, and endothelial dysfunction by determining the effect of a GRK2 inhibitor on blood pressure after clonidine/GRK2-inhibitor withdrawal and the effects of clonidine withdrawal-promoted endothelial dysfunction.

To the best of our knowledge, this is the first study to examine the effects of drug withdrawal in an experimental mouse model of simultaneous DM and hypertension. Previous studies in simultaneous models of hypertension and DM focused primarily on drug dosing schedules [33–35]. The main findings of this study are as follows: (1) clonidine treatment using an osmotic pump in DM mice inhibited

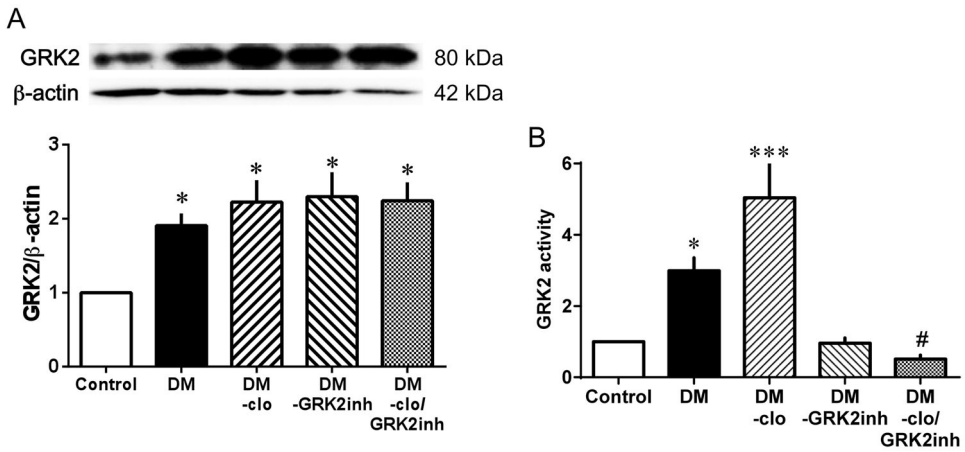

Fig. 3 Effects of drug withdrawal on GRK2 expression and activity 21 days after implantation of an osmotic pump in DM mice. **a** The expression levels of GRK2 were analyzed by western blotting in aortas from control, DM, clonidine-treated DM, GRK2-inhibitor-treated DM or clonidine/GRK2-inhibitor-cotreated DM mice and quantified by densitometric analysis. Data were normalized by β-actin protein levels. **b** Levels of GRK2 activity in aortas from control, DM, clonidine-treated DM, GRK2-inhibitor-treated DM, or clonidine/GRK2-inhibitor-cotreated DM mice. Values represent the mean ± SE; $n = 5$. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. Control. $^{\#}P < 0.05$ vs. DM mice

hypertension, but clonidine withdrawal in DM mice induced rebound hypertension, (2) a GRK2 inhibitor blocked the rebound hypertension induced by clonidine withdrawal in DM mice, and (3) a GRK2 inhibitor blocked clonidine withdrawal-induced endothelial dysfunction via activation of the Akt/eNOS/NO production pathway in DM mice. Taken together, these results suggest that the GRK2 inhibitor has a sustained effect and is beneficial in preventing vascular dysfunction by generating basal NO in DM mice. Acute withdrawal from clonidine in mice with simultaneous DM and hypertension results in activation of GRK2. This decreases basal NO production via the Akt/eNOS signaling pathway and, in turn, elevates arterial blood pressure to produce hypertension and impairs endothelial-dependent vascular function to produce endothelial dysfunction.

Various animal models that mimic characteristics of patients with DM have been generated by genetic, chemical, dietary, or surgical means or a combination of these techniques [36, 37]. Chemicals, such as STZ and Nic, have been demonstrated to induce experimental diabetes in rats and mice [13, 19, 22, 38]. In this study, DM was induced by using this method. We have previously reported that the characteristics of this model share a number of features with human DM: the model is characterized by stable hyperglycemia, hypertension, a lack of obesity, and similar levels of insulin secretion [13, 22]. As indicated in Fig. 2, the data from the present study are consistent with those previously reported [13, 22]. Therefore, the subjects within this model can be referred to as "DM mice".

Chronic diseases, such as hypertension and diabetes, are treated by using multidrug therapies, which are vulnerable to incidences of side-effects, poor patient compliance, and slow improvement of the disease state in patients. Clonidine is an antihypertensive drug belonging to the class of alpha-2 adrenergic receptor agonists and is generally used as an individual therapy to treat hypertension. However, it is a fourth-line drug treatment choice because of the risk of rebound hypertension caused by acute withdrawal [21]. Previously, we reported that clonidine stimulated NO production through activation of the Akt/eNOS pathway in the aorta, which is related to the endothelial-dependent vascular relaxation response [13, 22]. We consequently designed this study to suppress the negative effects of clonidine on endothelial function in mice with DM and hypertension.

A number of design options are available to modulate drug release from a dosage form. Osmotic systems utilize the principles of osmotic pressure for controlled delivery of drugs [39]. A previous study demonstrated that clonidine decreased blood pressure [24] and another showed that a low dose of a GRK2 inhibitor had no effect on blood pressure when an osmotic pump was used for drug infusion for 14 days in DM mice [13]. However, the influence of GRK2 on blood pressure after clonidine infusion has not been investigated. To the best of our knowledge, this is the first report showing that a GRK2 inhibitor altered clonidine withdrawal-induced rebound hypertension and endothelial-dependent vascular relaxation responses to activation of the Akt/eNOS pathway in DM mice. Significant and stable hypotensive effects were observed during clonidine infusion. Following cessation of this infusion period, blood pressure rapidly increased to DM levels at 15 days. Interestingly, clonidine/GRK2-inhibitor co-treatment did not result in hypertension after withdrawal in DM mice. Furthermore, plasma Ang II concentrations did not differ among DM, clonidine-treated DM, GRK2-inhibitor-treated DM, and clonidine/GRK2-inhibitor-cotreated DM mice at

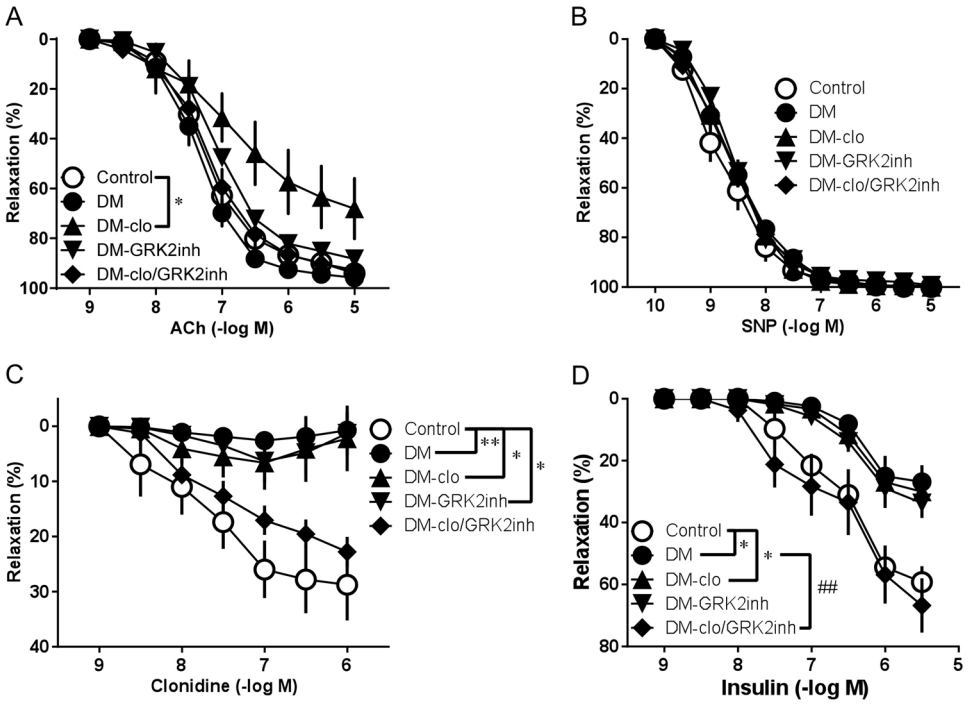

Fig. 4 Effects of drug withdrawal on vascular function 21 days after implantation of an osmotic pump in DM mice. Cumulative concentration–response curves to ACh (**a**; 10^{-9}–10^{-5} M), SNP (**b**; 10^{-10}–10^{-5} M), clonidine (**c**; 10^{-9}–10^{-6} M) and insulin (**d**; 10^{-9}–3×10^{-6} M) in aortas from control, DM, clonidine-treated DM, GRK2-inhibitor-treated DM or clonidine/GRK2-inhibitor-cotreated DM mice. Values represent the mean ± SE; $n = 5$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control. $^{\#\#}P < 0.01$ vs. DM mice

7 days after withdrawal. Thus, our findings suggest that clonidine-induced rebound hypertension immediately after withdrawal, and the GRK2 inhibitor suppressed this clonidine-induced rebound hypertension although the GRK2 inhibitor alone did not decrease blood pressure. Furthermore, the symptoms associated with clonidine withdrawal may not be dependent on plasma Ang II levels, which are related to its hypotensive effects. Alpha-2 adrenergic receptor lowers central sympathetic output and peripheral catecholamine release, which may prevent hypertension. Consequently, we were interested in the interactions among circulation catecholamine levels, clonidine, and GRK2 inhibition. However, we did not investigate catecholamine in this study because we focused on the interaction between blood pressure and the isolated aortic function. We plan to pursue this line of research in the future.

The present study showed continuous rebound hypertension from 1 day after clonidine withdrawal (15 days after implantation of an osmotic pump) in a simultaneous hypertension and DM model. In this context, we examined the underlying mechanisms by which a GRK2 inhibitor suppressed the rebound hypertension seen 7 days after clonidine withdrawal (21 days after implantation of the osmotic pump). We found that blood pressure, plasma glucose levels, plasma Ang II levels, aortic GRK2 levels, and aortic GRK2 activity were significantly increased in the DM mice relative to those in control animals. As shown in Fig. 3a, significant increases in GRK2 protein expression levels in the DM mice and drug-treated DM mice were observed relative to those in the controls. We previously reported increased GRK2 expression and activity and an increase in the plasma parameters in our DM mice [13, 14, 22]. Therefore, the present data indicated that clonidine withdrawal had an insignificant effect on GRK2 expression. Furthermore, as shown in Fig. 3b, significant increases in GRK2 activity in the DM mice and clonidine-treated DM mice were observed relative to those in the controls, which suggested that high GRK2 activation persisted unchanged in the DM mice and clonidine-treated DM mice. We also had reported that the DM mice had a markedly impaired response to clonidine and insulin via the Akt/eNOS signaling pathway [13, 22]. In the present study, clonidine withdrawal caused not only rebound hypertension and GRK2 activation but also endothelial dysfunction in the DM mice. In addition, clonidine/GRK2-inhibitor co-treatment suppressed this rebound hypertension and endothelial dysfunction (Fig. 4a) in the DM mice. Interestingly, GRK2 activation was inhibited by the GRK2 inhibitor, although its infusion was also stopped after 14 days. The IC_{50} value of this GRK2 inhibitor is ~130 μM [40]. However, in this study, we performed GRK2-inhibior infusion of 5.64 ng kg^{-1} day^{-1} for 2 weeks by using a subcutaneous osmotic pump. This concentration appears to be a very low dose, but the dose was selected on the basis of the results in our previous report [13]. Briefly, following long-term low-dose infusion of the GRK2 inhibitor had no effect on GRK2 expression, blood pressure, and endothelial function. The cytosolic serine/threonine kinase GRK2 is a critical regulator of GPCR and non-GPCR signaling. Once GRK2

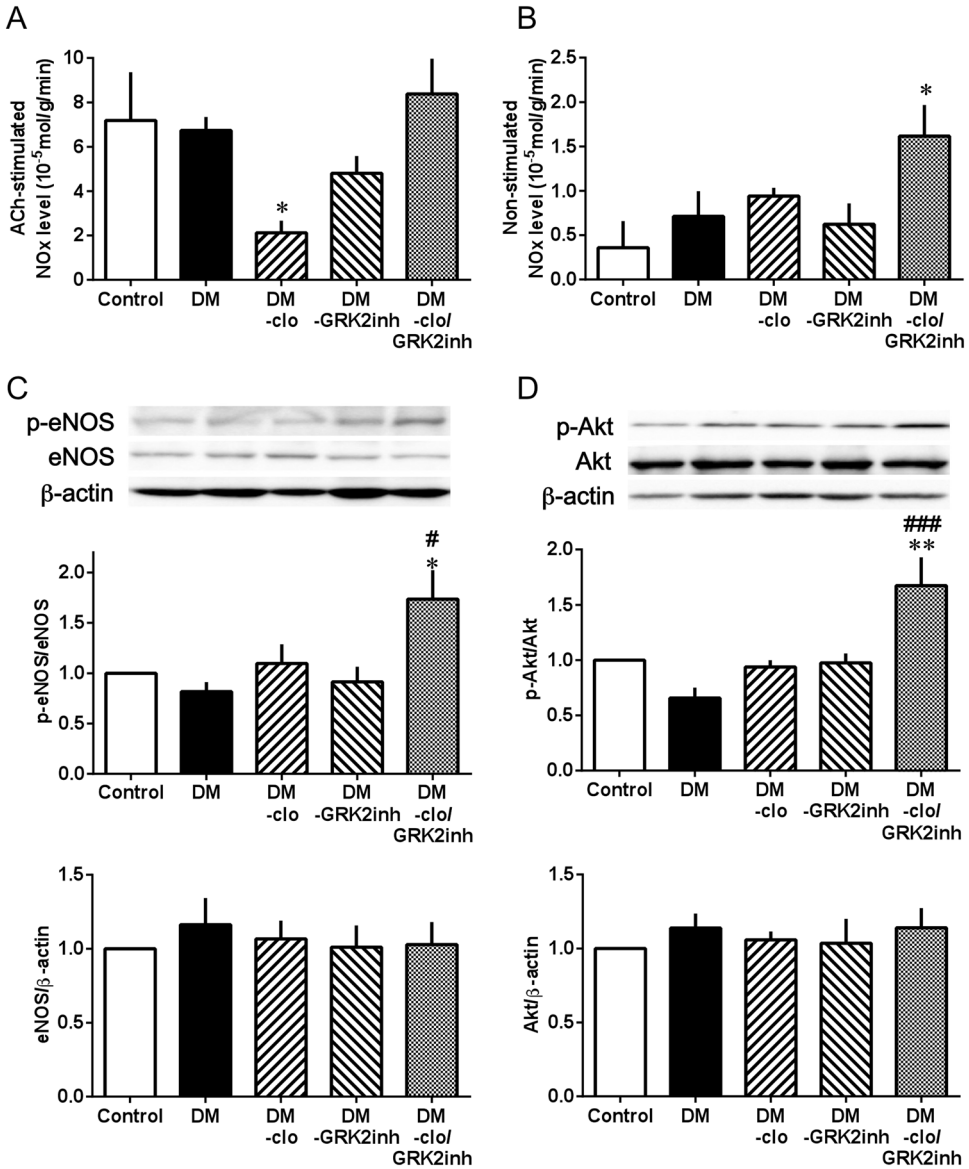

Fig. 5 Effects of drug withdrawal on NO production and Akt/eNOS activation 21 days after implantation of an osmotic pump in DM mice. ACh-stimulated **a** and non-stimulated **b** NOx production in aortas from control, DM, clonidine-treated DM, GRK2-inhibitor-treated DM or clonidine/GRK2-inhibitor-cotreated DM mice. **c** The expression levels of p-eNOS (Ser1177) and eNOS were analyzed by western blotting of aortic tissue from control, DM, clonidine-treated DM, GRK2-inhibitor-treated DM or clonidine/GRK2-inhibitor-cotreated DM mice and quantified by densitometric analysis. The data relating to eNOS activity are expressed as the ratio of p-eNOS (Ser1177)/total eNOS in the Control group. Total eNOS expression was normalized by β-actin protein levels. **d** The expression levels of p-Akt (Ser473) and Akt were analyzed by western blotting of aortic tissue from control, DM, clonidine-treated DM, GRK2-inhibitor-treated DM, or clonidine/GRK2-inhibitor-cotreated DM mice and quantified by densitometric analysis. The data relating to Akt activity are expressed as the ratio of p-Akt (Ser473)/total Akt in the Control group. Total Akt expression was normalized to β-actin protein levels. Values represent the mean ± SE; $n = 5$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. DM mice

is activated, many signaling pathways are stimulated. Recently, various additional effects of GRK2 have been discovered. These include promotion of DM and diabetic vascular complications. Both human and animal models of DM present upregulation of GRK2 protein expression and activation [13, 14, 18, 19]. We previously demonstrated that inhibition of GRK2 activation by the GRK2 inhibitor and GRK2 siRNA significantly improved vascular function and blood pressure [13, 14, 19]. In the present study, despite GRK2-inhibitor withdrawal for 2 weeks, GRK2 activation was inhibited (Fig. 3). Our results suggested that the reduced adverse GRK2 activity after low-dose infusion of GRK2 inhibitor could result from improved blood pressure and/or diabetic vascular complication in the Akt/eNOS

signaling pathway, and the GRK2 inhibitor showed an extended duration of action because of a long terminal half-life, which led to persistent inhibition of GRK2 activity. However, we still did not know how the inhibition of GRK2 activation was able to continue when the GRK2 inhibitor was no longer in the blood.

The attenuation of endothelial-dependent NO-mediated relaxation, referred to as endothelial dysfunction, has been demonstrated in DM and hypertension [22, 26] and has been suspected to contribute to excessive cardiovascular mortality. In addition, data on endothelial dysfunction in experimental models of drug withdrawal are scarce. In the present study, we investigated endothelial function in DM mice undergoing clonidine withdrawal. Our data show that endothelial functioning, as assessed by the vasrelaxation responses to ACh, clonidine, and insulin, are impaired in clonidine-treated DM mice. There are two broad categories of NO production [41]. The classical ACh-induced NO production pathway involved Ca^{2+}/calmodulin-dependent activation of eNOS. The second category of clonidine- and insulin-induced NO production involves eNOS phosphorylation via Akt activation. Early diabetic vascular endothelial dysfunction is characterized by the reduced second category-induced (Akt/eNOS signaling pathway) relaxation responses [22]. The data in Fig. 4a show no significant difference in ACh-induced relaxation between the control and DM mice. In contrast, clonidine- and insulin-induced relaxation responses were attenuated in the aorta from DM mice. Previously, we reported that the ACh-induced relaxation (a classical endothelium-dependent relaxation response) was not different between control and DM mice, but clonidine- and insulin-induced relaxation responses were decreased in the aortas of DM mice [13, 22]. The present data is in accordance with previous studies [13, 22]. To confirm that the abnormal response in aortas from DM mice to ACh was not due to a decreased response of vascular smooth muscle cells to NO, we demonstrated that the relaxing effect of the NO donor SNP was not impaired in all groups.

The impairment of endothelial-dependent relaxation in DM could be mediated via inhibition of eNOS-derived NO production. In this study, we found that clonidine withdrawal was associated with significant inhibition of ACh-stimulated NO production. Furthermore, we demonstrated increased NO production under non-stimulation conditions in aortas from clonidine/GRK2-inhibitor-cotreated DM mice. Additionally, clonidine- and insulin-induced relaxation responses were increased in aortas from clonidine/GRK2-inhibitor-co-treated DM mice. Clonidine and insulin activate Akt to mediate phosphorylation of eNOS (Ser1177) [22, 42, 43]. The activity of Akt is regulated by phosphorylation of Ser473 and Thr308 [44, 45]. Previous studies have demonstrated that clonidine activates Akt/eNOS signaling in DM aortas when co-administered with a GRK2 inhibitor [13, 14, 19]. In the present study, we demonstrated phosphorylation of Akt (Ser473) in the aorta 7 days after drug withdrawal in clonidine/GRK2-inhibitor-cotreated DM mice. Furthermore, the phosphorylation at Ser1177 by Akt represents a major mechanism for the activation of eNOS [43, 46, 47]. Constitutively expressed eNOS is a major source of NO production, and NO has an important role in the regulation of vasodilatation [47, 48]. It was previously shown that clonidine leads to phosphorylation of eNOS (Ser1177) in aortas [13, 22]. However, the authors concluded that "clonidine had the Akt phosphorylation leading to eNOS activation disturbed by GRK2 activation in DM aortas" [13]. The present study demonstrated for the first time that the effect of clonidine-dependent phosphorylation of eNOS (Ser1177) was continued by co-treatment with a GRK2 inhibitor even 7 days after clonidine withdrawal in DM mice. Consistent with these data, we found increased basal NO production in aortas from clonidine/GRK2-inhibitor-cotreated DM mice.

In conclusion, our study demonstrated a novel mechanism for regulation of rebound hypertension and vascular endothelial dysfunction resulting from clonidine withdrawal. Inhibition of GRK2 activation had a sustained effect despite GRK2-inhibitor withdrawal and conferred protection from rebound hypertension and endothelial dysfunction by increasing NO production via the Akt/eNOS signaling pathway. These findings revealed a role for GRK2 inhibition in hypertension associated with DM and provide an additional novel vascular mechanism for the protective effects of activated GRK2 inhibition.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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