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Coronary endothelial dysfunction prevented by small-conductance calcium-activated potassium channel activator in mice and patients with diabetes

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Abstract

Objective: To investigate coronary endothelial protection of a small-conductance calcium-activated potassium (SK) channel activator against a period of cardioplegic-hypoxia and reoxygenation (CP-H/R) injury in mice and patients with diabetes (DM) and those without diabetes (nondiabetic [ND]).

Methods: Mouse small coronary arteries/heart endothelial cells (MHECs) and human coronary arterial endothelial cells (HCAECs) were dissected from the harvested hearts of mice (n = 16/group) and from discarded right atrial tissue samples of patients with DM and without DM (n = 8/group). The SK current density of MHECs was measured. The in vitro small arteries/arterioles, MHECs, and HCAECs were subjected to 60 minutes of CP hypoxia, followed by 60 minutes of oxygenation. Vessels were treated with or without the selective SK activator NS309 for 5 minutes before and during CP hypoxia.

Results: DM and/or CP-H/R significantly inhibited the total SK currents of MHECs and HCAECs and significantly diminished the mouse coronary relaxation response to NS309. Administration of NS309 immediately before and during CP hypoxia significantly improved the recovery of coronary endothelial function, as demonstrated by increased relaxation responses to adenosine 5′-diphosphate and substance P compared with those seen in controls ($P < .05$). This protective effect was more pronounced in vessels from ND mice and patients compared with DM mice and patients ($P < .05$). Cell surface membrane SK3 expression was significantly reduced after hypoxia, whereas cytosolic SK3 expression was greater than that of the sham control group ($P < .05$).

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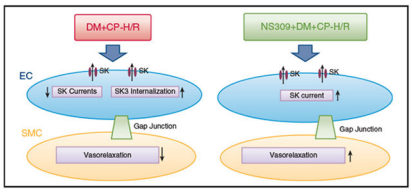
Conclusions: Application of NS309 immediately before and during CP hypoxia protects mouse and human coronary microvasculature against CP-H/R injury, but this effect is diminished in the diabetic coronary microvasculature. SK inhibition/inactivation and/or internalization/redistribution may contribute to CP-H/R-induced coronary endothelial and vascular relaxation dysfunction.

Graphical Abstract



SK activator–induced coronary arteriolar protection after CP-H/R.

Keywords

coronary microcirculation; diabetes; endothelial function; SK channels; hypoxia; ischemia; cardioplegia

Despite advances in myocardial protection strategies, cardioplegia (CP) and cardiopulmonary bypass (CPB) still induce postoperative coronary endothelial and vasomotor dysfunction, which can lead to coronary spasm, organ mal-perfusion, and cardiac dysfunction.[1,2] In particular, these changes are more profound in patients with diabetes mellitus (DM).[2–5] CP/CPB and DM are associated with endothelial nitric oxide (NO) synthase uncoupling, TXA-2:prostacyclin (PGI2) ratio imbalance, and down-regulation of endothelium-dependent hyperpolarization,[1,2,6] all of which contribute to compromised endothelial function, decreased coronary flow, and myocardial dysfunction. Therefore, protective strategies, such as pharmacologic modulation of CP solution are necessary to mitigate postoperative coronary endothelial dysfunction.

The small-conductance calcium-activated potassium (SK) channels are largely responsible for coronary arteriolar relaxation mediated by endothelium-dependent hyperpolarizing factor (EDHF).[6–11] There are 4 subtypes of SK channels—SK1, SK2, SK3, and SK4 (intermediate-conductance calcium-activated potassium [IK])—with SK3/SK4 present predominately in endothelial cells.[7–11] Inactivation of the endothelial SK channels contributes to CP/CPB-induced coronary microvascular endothelial dysfunction early after cardiac surgery.[8,9,11] Previous in vitro studies also demonstrated that administration of bradykinin resulted in SK activator–induced vascular endothelial protection in healthy animals following CP ischemia and reperfusion (CP-I/R)[11,12] or CP hypoxia and reoxygenation (CP-H/R).[9] Thus, we regard the SK channel modulator as a promising pharmaceutical target during CP-solution delivery. However, previous experiments were performed in isolated heart/vessels of healthy animals and did not accurately portray the clinical setting.[9,11,12] Actually, a majority of patients suffering from ischemic heart diseases have multiple comorbidities including DM. Importantly, we recently observed that DM was associated with SK channel inhibition and a greater degree of coronary microvascular endothelial dysfunction experienced by DM patients after CP/CPB and cardiac surgery.[13,14]

Intriguingly, previous studies only examined the direct effects of SK activators on SK channel currents of pig[9] and human coronary arterial endothelial cells (HCAECs)[13] and in vitro microvascular relaxation post–CP-I/R[8,14] or post–CP-H/R.[9] However, it remains unclear whether the pretreatment with SK activator–enriched CP solution may improve the recovery of coronary endothelial/microvascular function against a prolonged period of CP-I/R injury. Thus, we hypothesized that administration of SK activators immediately before and during CP hypoxia might differentially protect coronary vasculature in DM and nondiabetic (ND) animals and patients at some time after CP-H/R injury. By using the in vitro microvessel/cell models in mice[15] and humans[13,14] with DM and ND, we evaluated the therapeutic effects of the selective SK activator NS309 delivered immediately before and during CP hypoxia on the coronary endothelial protection and the recovery of vascular relaxation. To further investigate the potential cellular/molecular mechanisms responsible for NS309-induced protective effects, we examined the direct effects of NS309 on endothelial SK current density and coronary relaxation function; investigated possible cross-talk among NO, PGI2, and EDHF; and assessed the effects of CP-H/R on subcellular SK protein expression.

METHODS

Mice and Mouse Heart Tissue Harvesting

Sixteen genetically modified male mice (BKS.Cg-*Dock7*$^{m+/+}$ *Lepr*^{db}/J, age 10 weeks; The Jackson Laboratory, Bar Harbor, Me) exhibiting type 2 DM and obesity were used in this study. Sixteen C57BL/6J mice of the same age served as ND controls. All experiments were approved by the Institutional Animal Care and Use Committee of the Rhode Island Hospital (approval date: April 11,2019; internal reference no. 004410). Heparin was given intraperitoneally to prevent thrombosis. The mice were anesthetized using inhalant isoflurane, thoracotomy was performed, and the hearts were removed. The heart tissue was placed in cold Krebs buffer in preparation for in vitro microvascular study, preserved in cell culture medium for endothelial cell isolation, or stored in liquid nitrogen for molecular analysis.

Human Subjects and Atrial Tissue Harvesting

All procedures were approved by the Institutional Review Board of Rhode Island Hospital, Alpert Medical School of Brown University, and informed consent was obtained from all enrolled patients. The patients were then divided into 2 groups: ND patients with a normal hemoglobin A1c (HbA1c) value and no history of treatment for DM and DM patients with HbA1c ≥8.0.[13,14] Right atrial tissue samples were harvested from patients undergoing cardiac surgery before CP/CPB.

Endothelial Cell Isolation and Culture

MHECs were isolated from the harvested hearts of DM and ND mice,[16,17] and HCAECs from donors (DM and ND patients) were cultured as described previously.[13,14] The methodology is described in detail in Appendix E1.

Patch-Clamp Recording of Endothelial Cell Currents

The primarily cultured MHECs (passage 0) and HCAECs from DM and ND mice and patients were washed twice with Ca^{2+}–free DMEM and then incubated with 0.05% trypsin and 0.02% EDTA,[13,14,16,17] as described in detail in Appendix E1.

Isolated Microvessel Preparation

The mouse heart and human right atrial tissue samples were removed and immediately placed into cold (4°C) Krebs physiological saline solution, as described previously.[13–15]

CP-I/R Injury Simulation

In an attempt to simulate the nonoxygenated CP in the operating room, an in vitro CP model was used. The CP solution consisted of 110 mmol/L NaCl, 20 mmol/L KCl, 16 mmol/L $MgCl_2$, 1.5 mmol/L $CaCl_2$, and 10 mmol/L $NaHCO_3$. Vessels were subjected to 60-minute hypoxic CP at 15°C and then reoxygenated with *Krebs–Henseleit buffer* for 60 minutes at 37° C. Hypoxic conditions were induced by switching bubbling gas from 95% O_2/5% CO_2 to 95% N_2/5% CO_2, adjusted to a pH of 7.4 and a PCO_2 of 35 mm Hg.[18,19]

Evaluation of Microvascular Endothelial Function

At the end of CP-H/R, all vessels were preconstricted with endothelin-1, and the responses to endothelium-dependent vasodilators adenosine 5′-diphosphate (ADP; 10^{-9} M to 10^{-4} M) and substance P (10^{-12} M to 10^{-7} M) or endothelium-independent vasodilator sodium nitroprusside (SNP; 10^{-9} M to 10^{-4} M) were examined. We previously determined that the responses of coronary arterioles to ADP, substance P, and NS309 are endothelium-dependent.[3,4,8,13,15]

Cell Hypoxia Model

To examine the effects of the CP-H/R on SK channel expression, CP hypoxia was induced by incubating primary cultured MHECs and HCAECs in an airtight chamber with pure 95% N_2 and 5% CO_2 at 15°C for 60 minutes. The cells were transferred to a normoxic incubator (95% air and 5% CO_2) for 60 minutes of reoxygenation.

Membrane Protein Isolation

The cell membrane and cytosolic proteins of HCAECs were extracted using the Mem-PER Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific, Waltham, Mass). The methodology is described in detail in Appendix E1.

Immunoblotting

The methods for cell protein purification, Western blot analysis, and imaging quantification have been described previously[13,14] (see Appendix E1 for more details).

Experimental Protocols

Protocol 1: Direct effects of SK activator NS309 on endothelial SK currents.— Whole-cell currents of MHECs were recorded in both the ND and DM groups under normoxic conditions. The effects of NS309 on K^+ currents of MHECs were examined in the

presence or absence of the selective SK3-blocker apamin and the SK4-blocker TRAM34. Whole-cell currents of HCAECs were also recorded in both the ND and DM groups under normoxic and hypoxic conditions in the presence and absence of the selective SK3-blocker apamin and the SK4-blocker TRAM34.

Protocol 2: Microvascular reactivity

Protocol 2A: Direct effect of SK activator NS309 on the coronary relaxation response at baseline and after CP-H/R.: Mouse small coronary arteries with or without DM were precontracted with endothelin-1, followed by application of the selective SK activator NS309 (10^{-6} M) (n = 5/group). In these experiments, 2 vessels from the same coronary arterial bed were divided into 2 groups: a control (sham) group without CP-H/R and a group subjected to CP-H/R in the presence or absence of NS309.

Protocol 2B: Effect of CP-H/R on the vascular relaxation response.: In these experiments, vessels from the same coronary arterial or arteriolar bed were divided into 2 groups (n = 6–8/group). One group served as the control (sham) group without hypoxia, and the other group was subjected to CP-H/R. At the end of reoxygenation, ADP, substance P, and ANP-induced relaxation were examined in the precontracted vessels.

Protocol 2C: Effect of NS309-enriched CP treatment on the recovery of vascular relaxation following CP-H/R.: Vessels from mice and patients with DM and ND were treated 5 minutes immediately before hypoxia and during 60 minutes of CP hypoxia with the selective SK activator NS309 (10^{-7} M or 10^{-6} M). After 60 minutes of reoxygenation, ADP- and substance P-induced dose-dependent relaxation responses were measured in the precontracted vessels.

Protocol 2D: Effects of selective SK blockers on NS309-enriched CP-induced recovery of vascular function following CP-H/R.: Mouse vessels were treated with the selective SK activator NS309 (10^{-6} M) 5 minutes before hypoxia and during CP hypoxia in the presence of the selective SK3 blocker apamin (10^{-7} M) or apamin plus the selective SK4 (IK) blocker TRAM34 (10^{-5} M). At the end of CP-H/R, ADP- and substance P–induced dose-dependent relaxation responses were measured in the precontracted vessels.

Protocol 2E: Effects of L-N^G-nitroarginine methyl ester (L-NAME) and indomethacin on NS309 improved recovery of vascular function following CP-H/R.: Mouse vessels were treated with the selective SK activator NS309 (10^{-6} M) for 5 minutes before hypoxia and during 60 minutes of CP hypoxia in the presence of L-NAME (10^{-5} M) or indomethacin (10^{-4} M). At the end of CP-H/R, the substance P–induced dose-dependent relaxation response was measured in the precontracted vessels.

Protocol 3: Effects of CP-H/R on protein expression of endothelial SK channels

Protocol 3A: Effects of CP-H/R on total protein expression of endothelial SK channels.: MHECs and HCAECs with and without DM were divided into normoxic and hypoxic groups. The cells were collected after exposure to either normoxia or 60-minutes of

hypoxia with 60 minutes of reoxygenation. The total protein expression of SK channels was measured via Western blot analysis.

Protocol 3B: Effects of CP-H/R on subcellular protein expression of endothelial SK-channels.: The cell membrane and cytosolic proteins of HCAECs were extracted, and the subcellular protein expression of SK was measured by Western blot analysis.

Chemicals—ADP, apamin, endothelin-1, indomethacin, L-NAME, NS309, SNP, substance P, and TRAM34 were purchased from Sigma-Aldrich (St Louis, Mo).

Data Analysis—Data are expressed as mean ± SD. Microvessel data were analyzed using 2-way repeated-measures ANOVA with Bonferroni's post hoc test. A paired *t* test was used for the data analysis of mouse and patient characteristics. Categorical data were analyzed using the χ^2 test. One-way-ANOVA was used for protein expression. A *P* value <.05 was considered to indicate statistical significance. All statistical analyses were performed with Prism version 6 (GraphPad Software, La Jolla, Calif).

RESULTS

Mouse Characteristics

The mean body weight of genetically modified DM mice was higher than that of ND mice (46.18 ± 2.34 g vs 24.31 ± 1.64 g; $P = .0001$). The mean blood glucose level was also higher in the DM mice (541.4 ± 32.8 mg/dL vs 134.4 ± 27.3 mg/dL; $P = .0001$; n = 26/group).

Patient Characteristics

Patient characteristics are summarized in Table 1. All patients with hypertension were receiving an anti-hypertensive medication (β-blocker, calcium channel blocker, or angiotensin-converting enzyme inhibitor) and also receiving daily aspirin. The mean preoperative blood HbA1c level was 8.38 ± 0.41 in the DM patients and 5.6 ± 0.39 in the ND patients ($P < .0001$).

Decreased Endothelial SK Currents of MHECs With DM in Response to the SK Activator NS309

Administration of NS309 significantly increased the total K^+ currents of MHECs from ND and DM mice. Notably, the K^+ current response to NS309 was significantly lower in DM endothelial cells compared with ND endothelial cells (Figure 1, A–C). Subsequent application of apamin and TRAM34 abolished NS309-induced effects on K^+ currents in both types of cells (Figure 1, A–C).

Direct Effects of NS309 on Mouse Coronary Artery Relaxation Response

The application of NS309 (10^{-6} M) induced significant relaxation responses in small coronary arteries of mice with DM and ND (Figure 1, D), but this effect was more pronounced in the ND vessels at sham control conditions ($P < .05$ for both). After CP-H/R, the relaxation responses to NS309 were reduced for both ND and DM vessels, with a more pronounced decrease in the DM vessels ($P < .05$ for both).

Decreased Endothelial SK Currents of HCAECs With DM in Response to the SK Activator NS309 Under Normoxic and Hypoxic Conditions

Similar to the findings in MHECs, during normoxia, NS309 significantly increased the total K^+ currents in HCAECs in both the ND and DM groups; however, this effect was significantly ($P < .05$) lower in the DM group (Figure 2, A–C). NS309-sensitive K^+ currents were significantly ($P < .05$) reduced after hypoxia compared with those in normoxia in both groups, with a more profound reduction in the DM group (Figure 2, A–C). Application of the selective SK blocker apamin significantly ($P < .05$) diminished NS309-sensitive K^+ currents in both of ND and DM groups (Figure 2, D and E) and subsequent application of apamin plus the selective SK4 blocker TRAM34 further abolished NS309-sensitive K^+ currents in both the ND and DM groups (Figure 2, F and G). The apamin-sensitive currents and TRAM34-sensitive currents were significantly decreased after hypoxia in both the ND and DM groups compared with those seen in normoxic conditions ($P < .05$ for both), but this reduction was more pronounced in the DM group compared with the ND group ($P < .05$).

Recovery of Endothelium-Dependent Relaxation Function

Mouse coronary vasculature

Decreased endothelium-dependent relaxation response of mouse diabetic vessels in the sham control condition.: DM mice exhibited a significant reduction in the basal (sham control) relaxation response to the endothelium-dependent vasodilators ADP (Figure 3, A) and substance P (Figure 3, B) compared with ND (sham control) mice ($P < .05$).

Reduced endothelium-dependent relaxation response following CP-H/R in DM and ND mice.: On CP-H/R, a significant decrease in recovery of the mouse coronary relaxation response was observed for both ADP (Figure 3, A) and substance P (Figure 3, B) in both DM and ND conditions (sham control) ($P < .05$ for both). Notably, performing CP-H/R resulted in a more drastic reduction in recovery for the DM vessels ($P < .05$).

Treatment with NS309 improved the recovery of endothelium-dependent relaxation response following CP-H/R in DM and ND mice.: Treatment of mouse vessels with NS309 (10^{-7} M and 10^{-6} M) significantly enhanced the recovery of coronary endothelial function, demonstrating increased relaxation responses to ADP (Figure 3, A) and substance P (Figure 3, B) compared with CP-H/R alone ($P<.05$ for both). Furthermore, this protective effect was more pronounced in the vessels from ND mice compared with those of DM mice ($P < .05$). Interestingly, no significant differences in the relaxation response recovery to ADP (Figure 3, A) and substance P (Figure 3, B) were detected between 10^{-7} M NS309 and 10^{-6} M NS309 for both the ND and DM treatment groups ($P > .05$).

Effects of SK blockers on NS309-improved recovery of vascular function following CP-H/R.: Pretreating the mouse vessels with the selective SK blocker apamin (10^{-7} M) significantly ($P < .05$) reduced NS309-improved recovery of vascular relaxation in response to ADP (Figure 3, C) and substance P (Figure 3, D) following CP-H/R. Furthermore, pretreating the vessels with apamin plus the SK4 blocker TRAM34 (10^{-5} M) abolished NS309-improved recovery of vascular function after CP-H/R ($P < .05$).

Effects of L-NAME and indomethacin on NS309-improved recovery of vascular function following CP-H/R.: Pretreatment of mouse vessels with L-NAME (10^{-5} M) significantly reduced NS309-improved recovery of vascular relaxation in response to substance P (Figure 3, E) following CP-H/R. In contrast, pretreating the vessels with indomethacin (10^{-4} M) did not affect NS309-improved recovery of vascular function after CP-H/R ($P > .05$; Figure 3, F).

Human coronary microvasculature

Impaired endothelium-dependent relaxation response of coronary arterioles in DM patients in normoxic conditions.: In a similar pattern to the murine model, the basal (sham control) relaxation of human coronary arterioles in response to the endothelium-dependent vasodilator ADP (Figure 4, A) and substance P (Figure 4, B) was significantly reduced in the microvessels of DM patients compared with ND patients ($P < .05$).

Diminished recovery of endothelium-dependent relaxation following CP-H/R in microvessels of DM and ND patients.: A significant reduction in relaxation response recovery of human coronary arterioles for vasodilators ADP (Figure 4, A) and substance P (Figure 4, B) was observed after CP-H/R in both DM and ND microvessels ($P < .05$ for both). As seen in the murine model, implementation of CP-H/R resulted in a more stunted recovery for human DM microvessels compared with ND microvessels ($P < .05$; Figure 4, A and B).

NS309 enhanced the recovery of endothelium-dependent relaxation following CP-H/R in microvessels of DM and ND patients.: Recovery of the endothelium-dependent relaxation in response to ADP (Figure 4, A) and substance P (Figure 4, B) was drastically increased on administration of NS309 ($P < .05$). Just as in the murine model, this phenomenon was more evident in the ND microvessels. Finally, no significant change in the relaxation response recovery to ADP (Figure 4, A) or substance P (Figure 4, B) was observed between 10^{-7} M and 10^{-6} M NS309 for either the DM or ND treatment group.

Endothelium-Independent Relaxation Response Mouse coronary vasculature.—DM did not alter the basal relaxation response of perfused mouse small coronary arteries to the endothelium-independent vasodilator SNP compared with the ND sham control group (Figure 4, C). A significant reduction in recovery of the relaxation response to SNP was detected post-CP-H/R in both DM and ND mouse vessels ($P < .05$ for both); however, the difference in response to SNP between DM and ND vessels was statistically nonsignificant (Figure 4, C). Administration of NS309 drastically improved the relaxation response recovery of mouse small coronary arteries to SNP in both the DM and ND treatment groups, with no significant difference in response between the 2 groups.

Human coronary microvasculature.—The basal relaxation response of DM arterioles to SNP was drastically lower than that of ND patients ($P < .005$; Figure 4, D). CP-H/R significantly reduced the recovery of the microvascular relaxation response, and this effect was more pronounced in the DM condition (Figure 4, D). However, treatment with 10^{-7} M

or 10^{-6} M NS309 failed to alter the relaxation response recovery to SNP in both the DM and ND groups ($P > .05$).

Effect of CP-H/R on the subcellular protein expression of SK channels in HCAECs.: Under normoxic conditions in HCAECs, there was a significant decrease in SK3-protein expression in the cell-membrane extraction lysates in the DM group compared with ND groups (Figure 5, $P = .02$). CP-H/R further reduced the amount of SK3-protein in the cell-membrane fractions compared with that of the control group ($P = .01$). In contrast, CP-H/R failed to alter subcellular re-distribution of SK4 channels in the HCAECs for both the DM and ND groups (Figure 5, $P > .05$).

Effect of CP-H/R on the total protein expression of SK channels (whole-cell lysates).: In the whole-cell lysate preparation, there were no significant differences in total SK3 and SK4 protein expression in MHECs (Figure 6, A) and HCAECs (Figure 6, B) between the DM and ND groups at baseline ($P > .05$). CP-H/R slightly decreased total SK3 and SK4 protein expression in whole-cell lysates, but the difference failed to reach statistical significance in either MHECs (Figure 6, C and D) or HCAECs (Figure 6, E and F; $P > .05$).

DISCUSSION

In this study, we observed that administration of NS309 increased SK currents in both MHECs and HCAECs. In addition, DM decreased SK activity and current densities in MHECs and HCAECs under normoxic conditions. Evidently, the inhibition/inactivation of endothelial SK current density may be responsible for the reduced NS309-induced coronary relaxation under normoxic conditions. Importantly, our findings also show that CP-H/R significantly reduced endothelial SK currents in HCAECs, and that the reduction in SK currents after CP-H/R was more profound in DM cells compared with ND cells. These findings suggest that DM combined with CP-H/R further inhibit SK activity.

Depolarizing the cell membrane for a prolonged period during CP-I/R can cause endothelial damage, resulting in reduced endothelium-dependent relaxation.[1,8,20] Consistent with previous studies in pigs[9] and humans,[8,14] this study also found that NS309-induced vascular relaxation was diminished after CP-H/R in the precontracted vessels of mice, suggesting that CP-H/R inhibits endothelial SK channels. This impairment was more severe in the DM mice compared with the ND mice, confirming that DM combined with CP-H/R further inhibits SK activity.

A major novel finding of this study is that NS309 administered immediately before and during CP hypoxia protected coronary endothelial function via improved recovery of the endothelium-dependent relaxation responses to ADP and substance P. However, the protective effects were significantly less pronounced in DM vessels of the mice and patients. Moreover, the beneficial effects of NS309 were inhibited in the presence of SK blockers apa-min and TRAM34, suggesting the importance of both SK3 and SK4 channel activity in regulating the observed protective effects. These novel findings also imply that NS309, as an additive to CP solution, may restore EDHF-mediated endothelial function against CP-H/R injury.

To further investigate the potential cross-talk among NO, PGI2, and EDHF in NS309-induced vascular protection, we observed that the pretreatment with the NO synthase inhibitor L-NAME reduced the NS309-improved recovery of the relaxation response to substance P after CP-H/R, suggesting that NS309-induced endothelial protection acts partially through an endothelial NO pathway. This idea has been supported by recent experimental studies showing that NS309 increases endothelial NO synthase and NO release.[21,22] In contrast, pretreatment with indomethacin failed to affect NS309-induced protection, suggesting that the PGI2 pathway is not involved in NS309-induced protective effects. Administration of NS309 also improved recovery of the SNP-induced relaxation response following CP-H/R in mouse coronary vessels, but not in human vessels. This discrepancy may be due to the differing vessel bed responses to CP-H/R and comorbidities between animals and humans. The marginal beneficial effects of NS309 in DM vessels suggest that this therapeutic effect may be diminished in DM patients.

The present study also corroborates previously reported findings[9,14,15] by demonstrating no significant changes in the total SK protein expression in MHECs and HCAECs in DM compared with those in ND or post–CP-H/R conditions, suggesting that this effect is post-translational. Furthermore, this study is the first to report that CP-H/R caused significant SK3 redistribution from HCAEC plasma membranes to cytosol, suggesting that CP-H/R is associated with SK3 channel internalization/trafficking. Thus, CP-H/R–induced SK3 internalization/trafficking may play a role in the inhibition of SK channel activity and reduced recovery of the relaxation response to the potent SK activator NS309. On the other hand, a lack of significant changes in SK4 redistribution suggests that CP-H/R and/or DM also may affect SK channel activity/gating.

It is possible that the use of an in vitro microvascular perfusion system might not exactly replicate the in vivo conditions. Nonetheless, it is important to note that examining the therapeutic effects of NS309-enriched CP in the context of mouse and human coronary arterioles provides a crucial step between bench and bedside. Thus, the results of this study provide translational applications that can accelerate the development of novel therapies for patients afflicted with coronary endothelial dysfunction after CP/CPB and cardiac surgery.

In conclusion, the administration of the selective SK activator NS309 protects coronary microvasculature against CP-H/R–induced endothelial and vascular relaxation dysfunction, but this effect was diminished in the diabetic coronary vasculature of mice and humans. Inactivation and internalization/redistribution of SK channels may be the mechanisms responsible for CP-H/R–induced coronary endothelial dysfunction and impairment of EDHF-related microvascular relaxation (Figure 7) (Video 1).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Conflict of Interest statement

Sellke has served on the Data Safety and Monitoring Boards of clinical trials for Allergen and Octapharma, has been an advisory committee member for Stryker, and was Principal Investigator on a study sponsored by a grant from Bayer AG (Leverkusen, Germany). All other authors have nothing to disclose with regard to commercial support.

APPENDIX E1.: METHODS

Endothelial Cell Isolation and Culture

MHECs were isolated from the harvested heart of mice with or without type 2 DM, as described previously.[E1,E2] For each experiment, primary cultures were started simultaneously (a pool of 5 cases per group). MHECs (passage 0) were grown in DMEM with 20% FCS, pen/strep, 100 μg/mL heparin (Sigma-Aldrich), 100 μg/mL endothelial cell growth supplement (Biomedical Technologies, Stoughton, Mass), 1× nonessential amino acids, 2 mM L-glutamine, 1× sodium pyruvate, and 25 mM Hepes in a humidified incubator with 5% CO_2 at 37°C previous study.[E1,E2] Results were obtained in triplicate using 3 independent batches of isolation per group. HCAECs harvested from donors (patients) with and without diabetes (Lonza, Walkersville, Md) were cultured and grown in EGM-2 BulletKit medium (Lonza) in a humidified incubator with 5% CO_2 at 37°C according to the manufacturer's protocols.[E3,E4]

Patch-Clamp Recording of Endothelial Cell Currents

The primarily cultured MHECs (passage 0) from mice with DM and ND were washed twice with Ca^{2+}-free DMEM and then incubated with 0.05% trypsin and 0.02% EDTA for 1 to 2 minutes.[E3,E5] An Axopatch 200B amplifier and pClamp 10.6 (Molecular Devices, Foster City, Calif) were used to record and analyze K^+ currents of MHECs in the whole-cell configuration in the voltage-clamp mode. The bath solution contained 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Hepes, and 30 mM glucose (pH 7.4), at 36°C for MHECs and room temperature for HCAECs. The patch pipette resistance was 1 to 3 MΩ, and the pipette solution contained 110 mM K-aspartate, 20 mM KCl, 1 mM $MgCl_2$, 8.5 mM $CaCl_2$, 10 mM Hepes, 8 mM NaCl, 0.01 mM niflumic acid, and 10 mM BAPTA (pH 7.2, with calculated free Ca^{2+} 400 nM). The cells were examined every 5 seconds at the holding potential of -50 mV by 150-ms test pulses between −100 and +100 mV in 20-mV increments. The sampling rate was 10 kHz, with low-pass filtering set at 2 kHz. The effect of selective SK channel activator NS309 (10^{-6} M) on the whole-cell K^+ currents was examined. The specificity of NS309 was confirmed by simultaneous application of the selective SK (SK2/SK3) blocker apamin (10^{-7} M) and the SK4 (IK) blocker TRAM34 (10^{-5} M).

Isolated Microvessel Preparation

The mouse heart (n = 6-8/group) and human right atrial tissue samples (n = 8/group) were removed and immediately placed into cold (4°C) Krebs physiological saline solution consisting of NaCl, 119.0 mmol/L; $NaHCO_3$, 25.0 mmol/L; KCl, 4.6 mmol/L; KH_2PO_4, 1.2 mmol/L; $MgSO_4$, 1.2 mmol/L; $CaCl_2$, 1.8 mmol/L; and glucose, 11.0 mmol/L. The mouse small coronary (LAD) arteries (70-120 μm internal diameter),[E1,E2,E6] and human coronary arterioles[E7–E11] (80-150 μm internal diameter) were dissected using a 10× to 60× dissecting microscope (Olympus, Tokyo, Japan). Microvessel studies were performed in vitro in a pressurized (40 mm Hg) no-flow state using video microscopy as described previously.[E1,E2,E6–E11]

Membrane Protein Isolation

The cell membrane and cytosolic proteins of HCAECs were extracted using the Mem-PER Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific). Cells in the growth media were resuspended by scraping the cells off the surface of the plate. The harvested cell suspension was centrifuged at 300 × *g* for 5 minutes. The resulting cell pellet was washed with 3 mL of cell wash solution and then centrifuged at 300 × *g* for 5 minutes. The supernatant was carefully removed and discarded. The cell pellet was vortexed and incubated with 0.75 mL of permeabilization buffer for 10 minutes at 4°C, after which the permeabilized cells were centrifuged for 15 minutes at 16,000 × *g*. The supernatant containing cytosolic proteins was carefully transferred to a new tube. The pellet was resuspended by adding 0.5 mL of solubilization buffer, incubated at 4°C for 30 minutes, and then centrifuged at 16,000 × *g* for 15 minutes at 4° C to obtain the supernatant containing sol- ubilized membrane and membrane-associated proteins.

Immunoblotting

The methods for whole-cell protein purification, Western blotting, and imaging quantification have been described previously.[E3,E4] Membranes were incubated overnight at 4°C with primary antibodies against SK3 and SK4 (IK-1; Alomone Labs, Jerusalem, Israel). After washing with TBST, membranes were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase. All membranes were also incubated with glyceraldehyde- 3-phosphate or beta-actin (Cell Signaling Technology, Dan- vers, Mass) as loading controls.

RESULTS

Dose-and Endothelium-Dependent Relaxation Responses to ADP and Substance P in Mouse Small Coronary Arteries

Dose- and endothelium-dependent relaxation responses to ADP (Figure E1, A and B) and substance P (Figure E1, C and D) of mouse diabetic vessels were significantly decreased under the sham control condition and following CP-H/R in DM and ND mice. Treatment with the SK activator NS309 improved the recovery of dose- and endothelium-dependent relaxation response following CP- H/R in DM and ND mice.

Dose- and Endothelium-Dependent Relaxation Responses to ADP and Substance P in Human Coronary Arterioles

Dose- and endothelium-dependent relaxation responses of coronary arterioles to ADP (Figure E2, A and B) and substance P (Figure E2, C and D) in patients with DM were impaired at basal conditions and following CP-H/R in microvessels of patients with DM and ND. The selective SK activator NS309 enhanced the recovery of the dose- and endothelium-dependent relaxation following CP-H/R in microvessels of patients with DM and ND.

Dose-Dependent and Endothelium-Independent Relaxation Responses to SNP

In the mouse coronary vasculature, there were no significant differences in dose-dependent and endothelium-independent basal relaxation responses to SNP in mouse small coronary arteries between the DM and ND groups (Figure E3, A and B). However, the dose-dependent and endothelium-independent relaxation responses to SNP were significantly reduced after CP-H/R in both DM and ND mouse vessels ($P < .05$ for both). Administration of NS309 drastically improved the dose-dependent relaxation response recovery of mouse small coronary arteries to SNP in both the DM and ND treatment groups (Figure E3, A and B).

In the human coronary microvasculature, dose- and endothelium-independent basal relaxation responses of coronary arterioles to SNP in patients with DM were impaired compared with patients with ND (Figure E3, C and D). Dose- and endothelium-independent relaxation responses of coronary arterioles to SNP were further reduced following CP-H/R in microvessels of patients with DM and ND; however, treatment with NS309 failed to alter the dose-relaxation response recovery to SNP in both the DM and ND groups ($P > .05$; Figure E3, C and D).

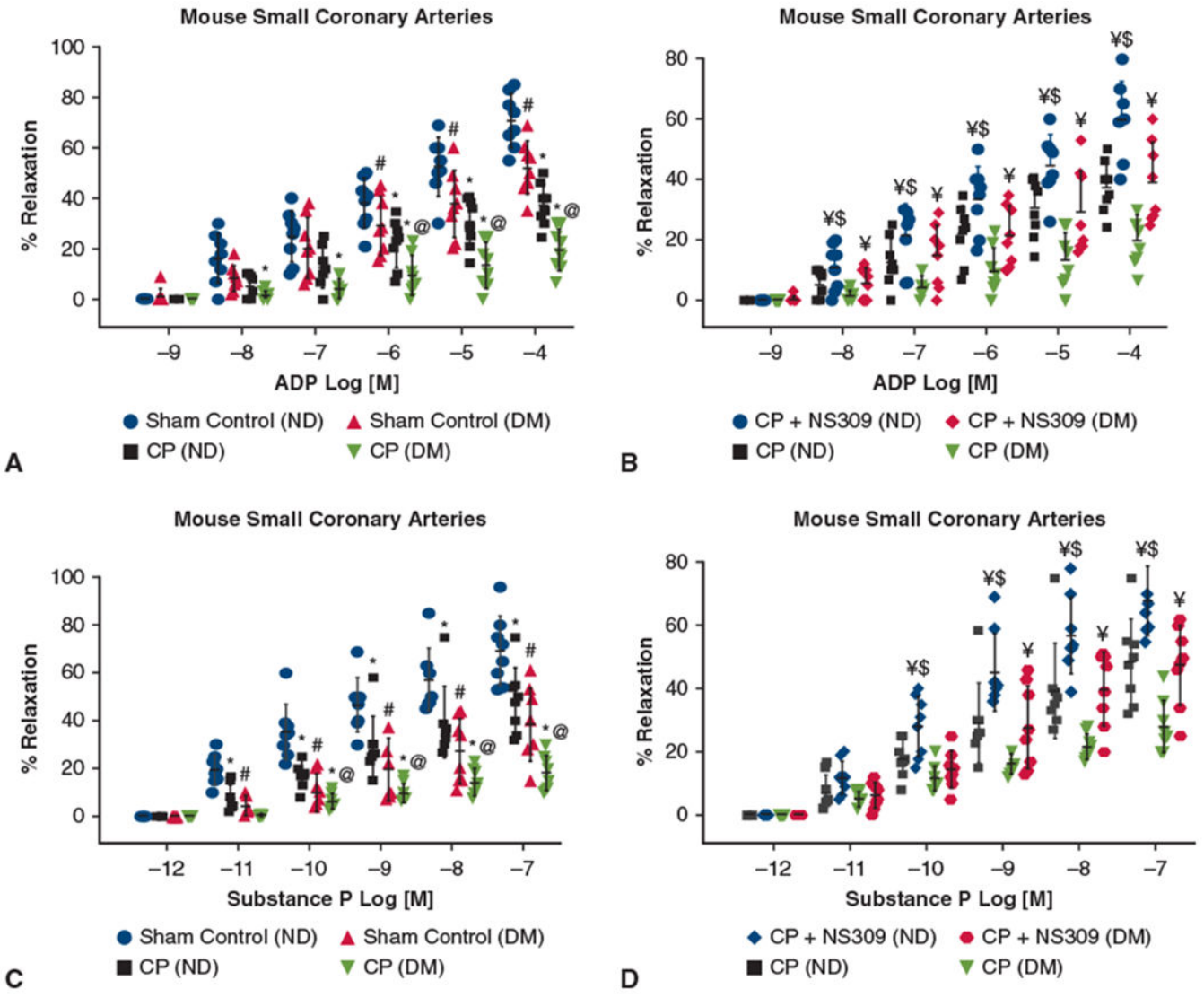

FIGURE E1.

A, Dose-dependent relaxation response of mouse small coronary arteries to the endothelium-dependent vasodilator adenosine 5′-diphosphate (*ADP*; 10^{-9} M to 10^{-4} M) at baseline (sham control groups) and recovery of the relaxation response after cardioplegic hypoxia and reoxygenation (*CP-H/R*) from the diabetic (*DM*) and nondiabetic (*ND*) groups. Sham control (DM), sham control in DM vessels; sham control (ND), sham control in ND vessels; CP (DM), CP-H/R in DM vessels; CP (ND), CP-H/R in ND vessels. B, Recovery of mouse coronary relaxation to ADP (10^{-9} M to 10^{-4} M) after 60 minutes of CP-H/R in the absence or presence of NS309 (10^{-7} M) pretreatment plus NS309 (10^{-7} M)-enriched CP in the DM and ND groups. CP + NS309 (DM), NS309 pretreatment plus NS309-enriched CP in the DM group, CP + NS309 (ND), NS309 pretreatment plus NS309-enriched CP in the ND group. C, Dose-dependent relaxation response of mouse small coronary arteries to the endothelium-dependent vasodilator substance P (10^{-12} M to 10^{-7} M) at baseline (sham controls) and recovery of the relaxation response after CP-H/R from ND and DM mice. D, Recovery of mouse coronary relaxation to substance P (10^{-12} M to 10^{-7} M) after 60 minutes

of CP in the absence or presence of NS309 (10^{-7} M) pretreatment plus NS309 (10^{-7} M)-enriched CP in the DM and ND groups. $^{*}P < .05$ vs sham control (ND) or sham control (DM); $^{\#}P < .05$ vs sham control (ND); $^{@}P < .05$ vs CP (ND); $^{¥}P < .05$ vs CP (ND) or CP (DM); $^{\$}P < .05$ vs CP + NS309 (ND). Data are mean ± SD. n = 8/group.

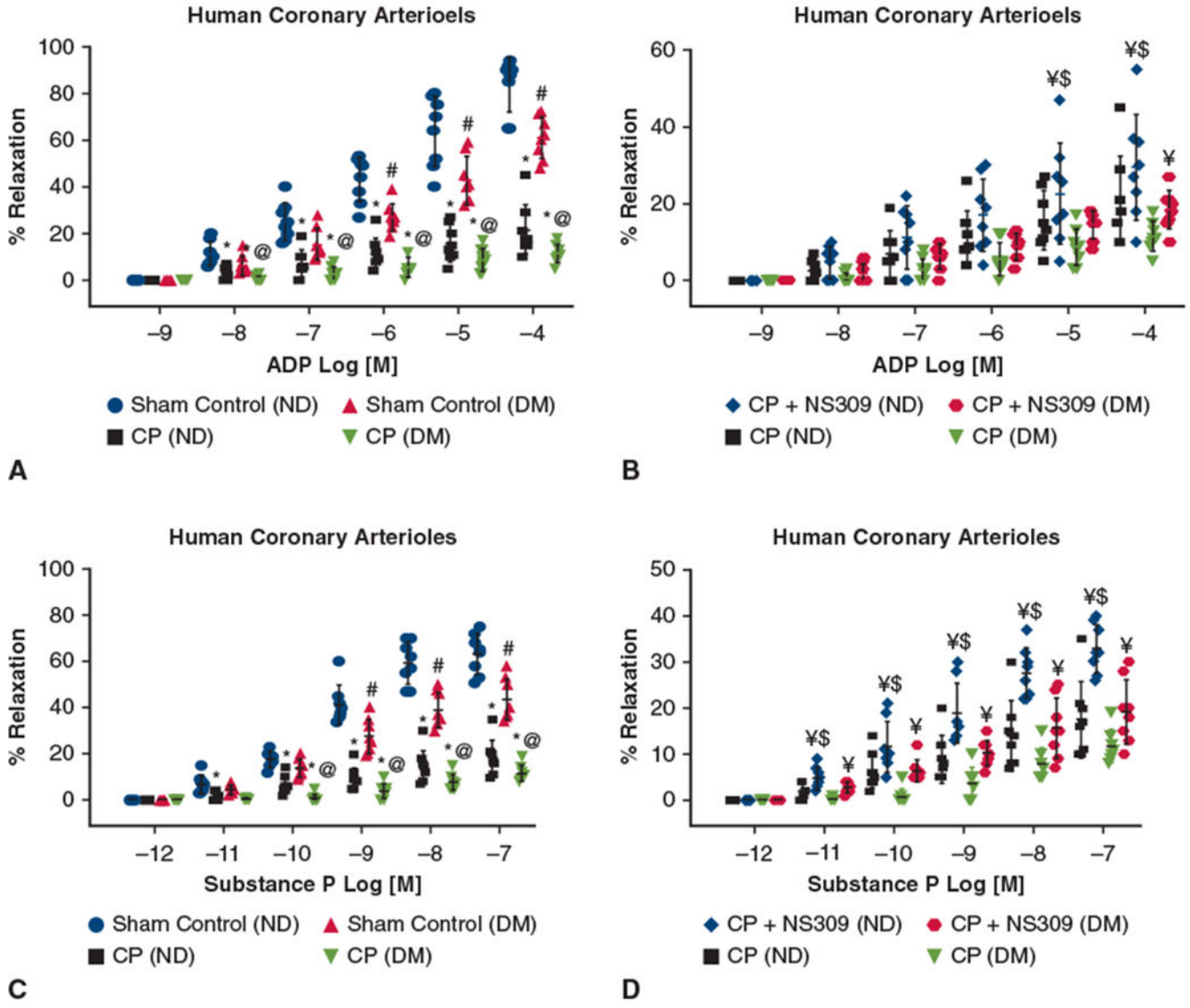

FIGURE E2.

A, Dose-dependent relaxation response of human coronary arterioles to adenosine 5′-diphosphate (*ADP*; 10^{-9} M to 10^{-4} M) at baseline (sham controls) and recovery of the relaxation response following 60 minutes of cardioplegic hypoxia and reoxygenation (*CP-H/R*) from diabetic (*DM*) and nondiabetic (*ND*) patients. Sham control (DM), sham control in DM vessels; sham control (ND), sham control in ND vessels; CP (DM), CP-H/R in DM vessels; CP (ND), CP-H/R in ND vessels. B, Recovery of human coronary arteriolar relaxation to ADP (10^{-9} M to 10^{-4} M) after 60 minutes of CP-H/R in the absence or presence of NS309 pretreatment plus NS309 (10^{-7} M)-enriched CP in the DM and ND

groups. CP + NS309 (DM), NS309 pre-treatment plus NS309-enriched cardioplegia in the DM group; CP + NS309 (ND), NS309 pretreatment plus NS309-enriched cardioplegia in the ND group. C, Dose-dependent relaxation response of human coronary arterioles to substance P (10^{-12} M to 10^{-7} M) at baseline (sham control) and recovery of the relaxation response following 60 minutes of CP-H/R from the DM and ND patients. D, Recovery of human coronary arteriolar relaxation to substance P (10^{-12} M to 10^{-7} M) after 60 minutes of CP-H/R in the absence or presence of NS309 (10^{-7} M) pretreatment plus NS309 (10^{-7} M)-enriched CP in DM and ND patients. *P < .05 vs sham control (ND) or sham control (DM); #P < .05 vs sham control (ND); @P < .05 vs CP (ND); ¥P < .05 vs CP (ND) or CP (DM); $$P$ < .05 vs CP + NS309 (ND). Data are mean ± SD. n = 8/group.

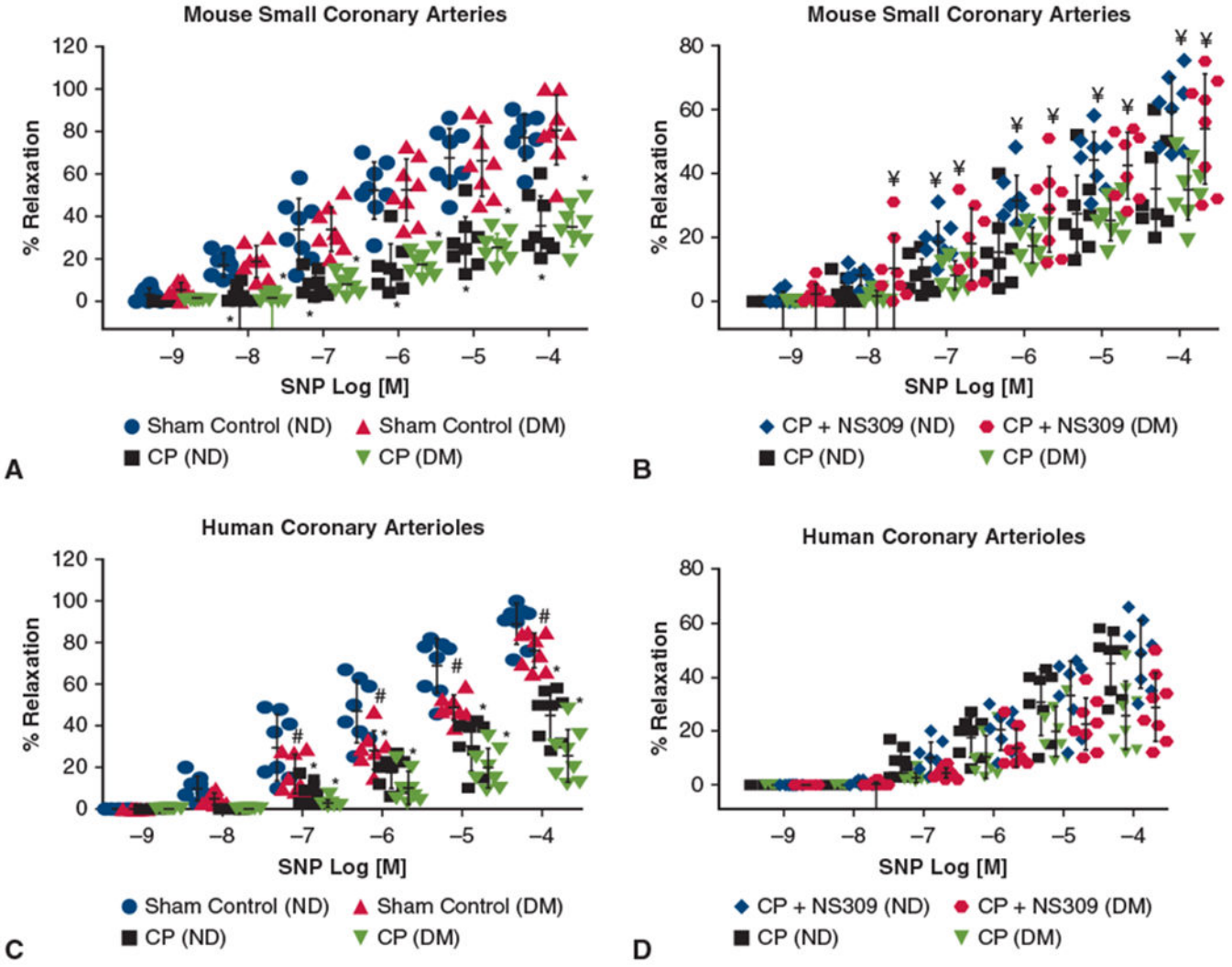

FIGURE E3.

A, Dose-dependent relaxation response of mouse small coronary arteries to the endothelium-dependent vasodilator sodium nitroprusside (*SNP*) (10^{-9} M to 10^{-4} M) at baseline (sham groups) and recovery of the relaxation response after cardioplegic hypoxia and reoxygenation (*CP-H/R*) from diabetic (*DM*) and nondiabetic (*ND*) groups. Sham control

(DM), sham control in DM vessels; sham control (ND), sham control in ND vessels; CP (DM), CP-H/R in DM vessels; CP (ND), CP-H/R in ND vessels. B, Recovery of mouse coronary relaxation to sodium nitroprusside (SNP; 10^{-9} M to 10^{-4} M) after 60 minutes of CP-H/R in the absence or presence of NS309 (10^{-7} M) pretreatment plus NS309-enriched CP in the DM and ND groups. CP + NS309 (DM), NS309 pretreatment plus NS309-enriched CP in the DM group. CP + NS309 (ND), NS309 pretreatment plus NS309-enriched CP in the ND group. C, Dose-dependent relaxation response of human coronary arterioles to SNP (10^{-9} M to 10^{-4} M) at baseline (sham control) and recovery of the relaxation response following 60 minutes of CP-H/R from ND and DM patients. D, Recovery of human coronary arteriolar relaxation to SNP (10^{-9} M to 10^{-4} M) after 60 minutes of CP-H/R in the absence or presence of NS309 pretreatment in DM and ND patients. CP represents CP-H/R. $^{*}P < .05$ vs sham control (ND) or sham control (DM); $^{\#}P < .05$ vs sham control (ND); $^{@}P < .05$ vs CP (ND); $^{¥}P < .05$ vs CP (ND) or CP (DM); $^{\$}P < .05$ vs CP + NS309 (ND) Data are mean ± SD. n = 6 to 8/group for mouse groups, n = 8/group for human groups.

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Abbreviations and Acronyms

ADP	adenosine 5′-diphosphate
CP	cardioplegia
CPB	cardiopulmonary bypass
CP-H/R	cardioplegic hypoxia and reoxygenation
CP-I/R	cardioplegic ischemia and reperfusion
DM	diabetes mellitus
EDHF	endothelium-dependent hyperpolarizing factor
HCAEC	human coronary artery endothelial cell
HbA1c	hemoglobin A1c
IK	intermediate-conductance calcium-activated potassium
L-NAME	L-N^G-nitroarginine methyl ester
MHEC	mouse heart endothelial cell
ND	nondiabetic
NO	nitric oxide
PGI2	prostacyclin
SK	small-conductance calcium-activated potassium
SNP	sodium nitroprusside

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PERSPECTIVE

Inclusion of the selective SK activator NS309 in cardioplegia protects the coronary microvasculature against CP-H/R-induced endothelial dysfunction, but this effect is reduced in the diabetic coronary microvasculature of mice and humans. SK3 internalization and diabetic inhibition of SK channels may be the mechanisms responsible for the DM and CP-H/R-induced coronary endothelial dysfunction and decreased coronary microvascular relaxation.

CENTRAL MESSAGE

Inclusion of SK channel activator NS309 in cardioplegia protects mouse and human coronary microvasculature against CP-H/R injury, but this beneficial effect is significantly diminished in the diabetic vasculature.

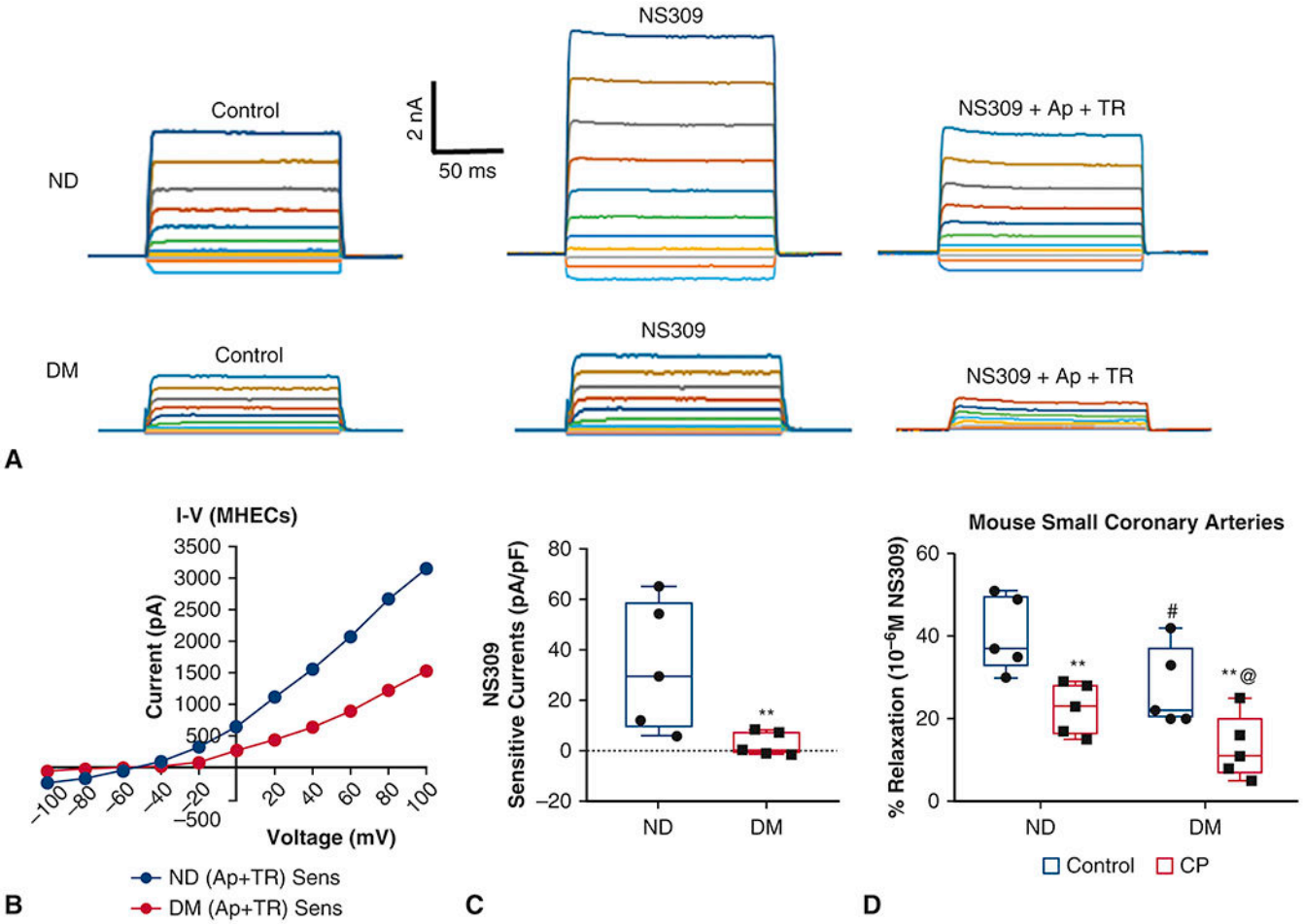

FIGURE 1.

Selective small-conductance calcium-activated potassium (SK) channels activator NS309 increased whole-cell K^+ currents in mouse heart endothelial cells (*MHECs*) of nondiabetics (*ND*) and diabetics (*DM*), which was blocked by simultaneous coapplication of the selective SK2/SK3 blocker apamin (*AP*) and SK4 blocker TRAM34 (*TR*). A, Representative current traces were obtained under control conditions in the presence of NS309 (10^{-6} M) and AP (10^{-7} M) plus TR (10^{-5} M). B, Current–voltage (I-V) plot of the blocker-sensitive currents shown in A, demonstrating less current in DM cells compared with ND cells. C, Cumulative bar graph of NS309-activated currents at +100 mV showing that the amplitude of the SK currents in response to the SK activator NS309 was significantly reduced in DM MHECs compared with ND MHECs. n = 5/group. **P* < .05. D, Relaxation response of mouse small coronary artery to NS309 (10^{-6} M) at baseline (sham control) and after cardioplegic hypoxia/reoxygenation (CP-H/R). n = 5/group, #*P* < .05 vs ND; ***P* < .001 vs control; @*P* < .05 vs ND.

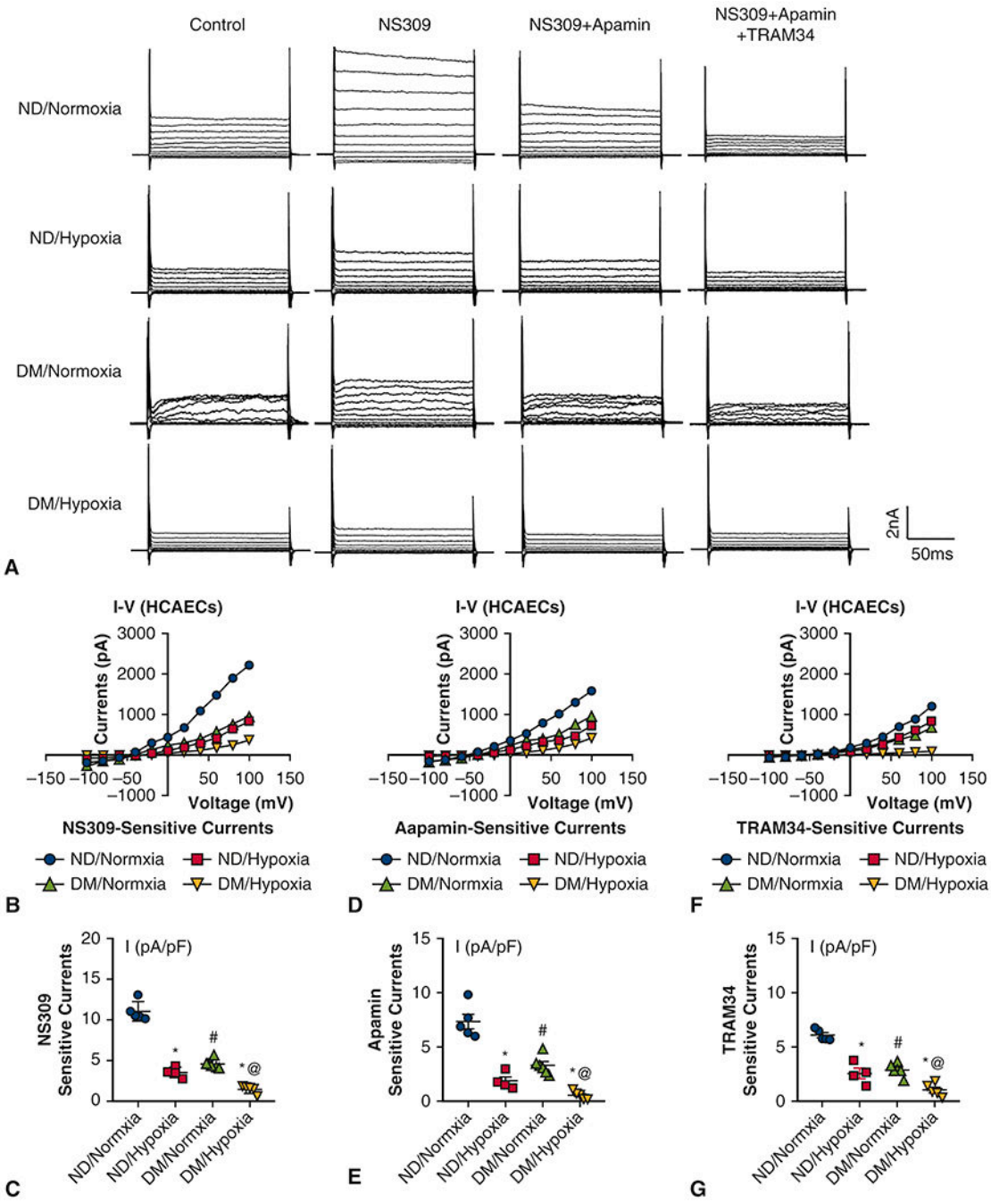

FIGURE 2.

The effect of selective small-conductance calcium-activated potassium (SK) channel activator NS309 on whole-cell K^+ currents in the human coronary artery endothelial cells (*HCAECs*) of the nondiabetic (*ND*) and diabetic (*DM*) groups under normoxic and hypoxic conditions. A, Representative current traces obtained from ND and DM cells under normoxic and hypoxic conditions in the presence of NS309 (10^{-6} M) and apamin (AP; 10^{-7} M) or AP + TRAM34 (TR; 10^{-5} M) cotreatment. B, Current–voltage (*I-V*) plot of the NS309-sensitive currents. C, Cumulative bar graph of NS309-activated currents (pA/pF) at

+100 mV. D, I-V plot of the AP-sensitive currents. E, Cumulative bar graph of AP-sensitive currents (pA/pF) at +100 mV. F, I-V plot of TR-sensitive currents. G, Cumulative bar graph of TR-sensitive currents (pA/pF) at +100 mV. n = 4 to 6/group. *$P < .05$ vs ND/hypoxia or DM/hypoxia; $^{\#}P < .05$ vs ND/normoxia; $^{@}P < .05$ vs ND/hypoxia.

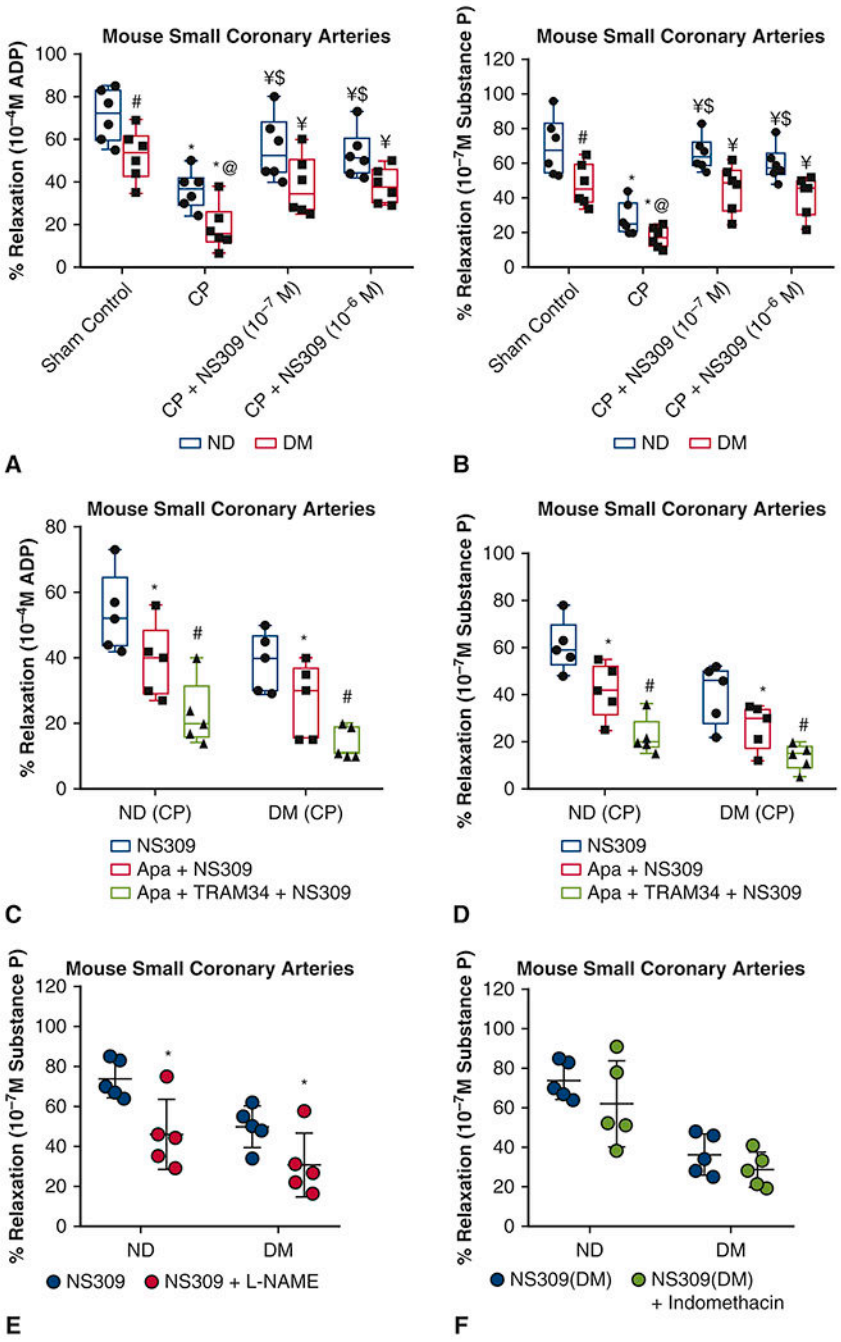

FIGURE 3.

A, Bar graphs showing recovery of the relaxation response of mouse small coronary arteries to adenosine 5′-diphosphate (*ADP*; 10^{-4} M) after 60 minutes of cardioplegic (CP) hypoxia and reperfusion (CP-H/R) in sham controls, CP alone, CP + NS309 (10^{-7} M), and CP + NS309 (10^{-6}M) groups in diabetic (*DM*) and nondiabetic (*ND*) vessels. CP represents CP-H/R. B, Bar graphs showing recovery of the relaxation response of mouse small coronary arteries to substance P (10^{-7} M) in sham controls, CP alone, CP + NS309 (10^{-7} M), and CP + NS309 (10^{-6} M) groups in DM and ND vessels. **P* < .05 vs sham control (ND) or sham

control (DM); #$P < .05$ vs sham control (ND); @$P < .05$ vs CP (ND); $^{U}P < .05$ vs CP (ND) or CP (DM); \$$P < .05$ vs CP + NS309 (ND). Data are mean ± SD. n = 6 to 8/group. C and D, Effects of small conductance calcium-activated potassium channels (SK) 3 blocker apamin (*Apa*) (10^{-7} M) alone (C) and Apa + SK4 blocker TRAM34 (10^{-5} M) (D) pretreatment on NS309-induced recovery of the relaxation response to ADP and substance P in mouse small coronary artery after 60 minutes of cardioplegic hypoxia and reoxygenation (CP) in the DM and ND groups; n = 5/group, data are mean ± SD, *$P < .05$ vs NS309 alone; #$P < .05$ vs NS309 + Apa. E, Effects of pretreatment with L-NAME on NS309-induced recovery of the relaxation response to substance P in mouse small coronary artery after 60 minutes of cardioplegic hypoxia and reperfusion in the DM and ND groups, n = 5/group, *$P < .05$ vs NS309 alone. F, Effects of pretreatment with indomethacin on NS309-induced recovery of relaxation response to substance P in mouse small coronary artery after 60 minutes of cardioplegic hypoxia and reperfusion in the DM and ND groups. n = 5/group. Data are mean ± SD.

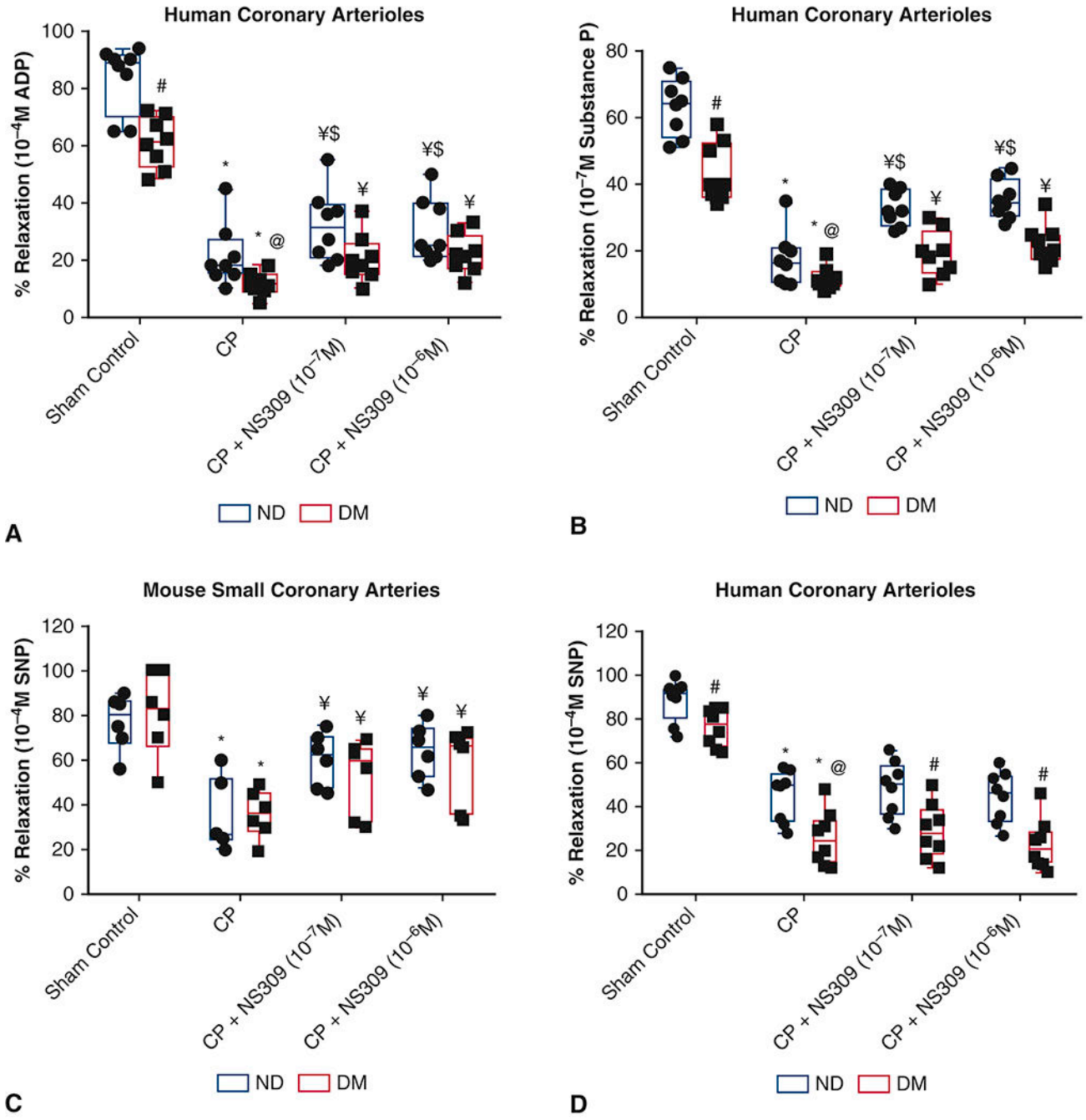

FIGURE 4.

A, Bar graphs showing recovery of the relaxation response of human coronary microvessels to adenosine 5′-diphosphate (10^{-4} M) after 60 minutes of cardioplegic (CP) hypoxia and reperfusion (CP-H/R) in sham controls, CP alone, CP + NS309 (10^{-7} M), and CP + NS309 (10^{-6} M) groups in the diabetic (*DM*) and nondiabetic (*ND*) vessels. CP represents CP-H/R. B, Bar graphs showing recovery of the relaxation response of human coronary microvessels to substance P(10^{-7} M) in sham controls, CP alone, CP + NS309 (10^{-7} M), and CP + NS309 (10^{-6}M) groups in DM and ND vessels. *$P < .05$ vs sham control (ND) and sham control

(DM); #$P < .05$ vs sham control (ND); @$P < .05$ vs CP (ND); $^{U}P < .05$ vs CP (ND) or CP (DM); \$$P < .05$ vs CP + NS309 (ND). Data are mean ± SD. n = 8/group. C, Bar graphs showing recovery of the relaxation response of mouse coronary vessels to SNP (10^{-4} M) in sham controls, CP alone, CP + NS309 (10^{-7} M), and CP + NS309 (10^{-6} M) groups in DM and ND vessels. *$P < .05$ vs sham control (ND) or sham control (DM). $^{U}P < .05$ vs CP (ND) or CP (DM). n = 6 to 8/group. D, Bar graphs showing recovery of the relaxation response of mouse coronary vessels to SNP (10^{-4} M) in sham controls, CP alone, CP + NS309 (10^{-7} M), and CP + NS309 (10^{-6} M) groups in DM and ND vessels. *$P < .05$ vs sham control (ND) or sham control (DM); #$P < .05$ vs sham control (ND); @$P < .05$ vs CP (ND); $^{U}P < .05$ vs CP (ND) or CP (DM); data are mean ± SD, n = 8/group. **SNP,** Sodium nitroprusside.

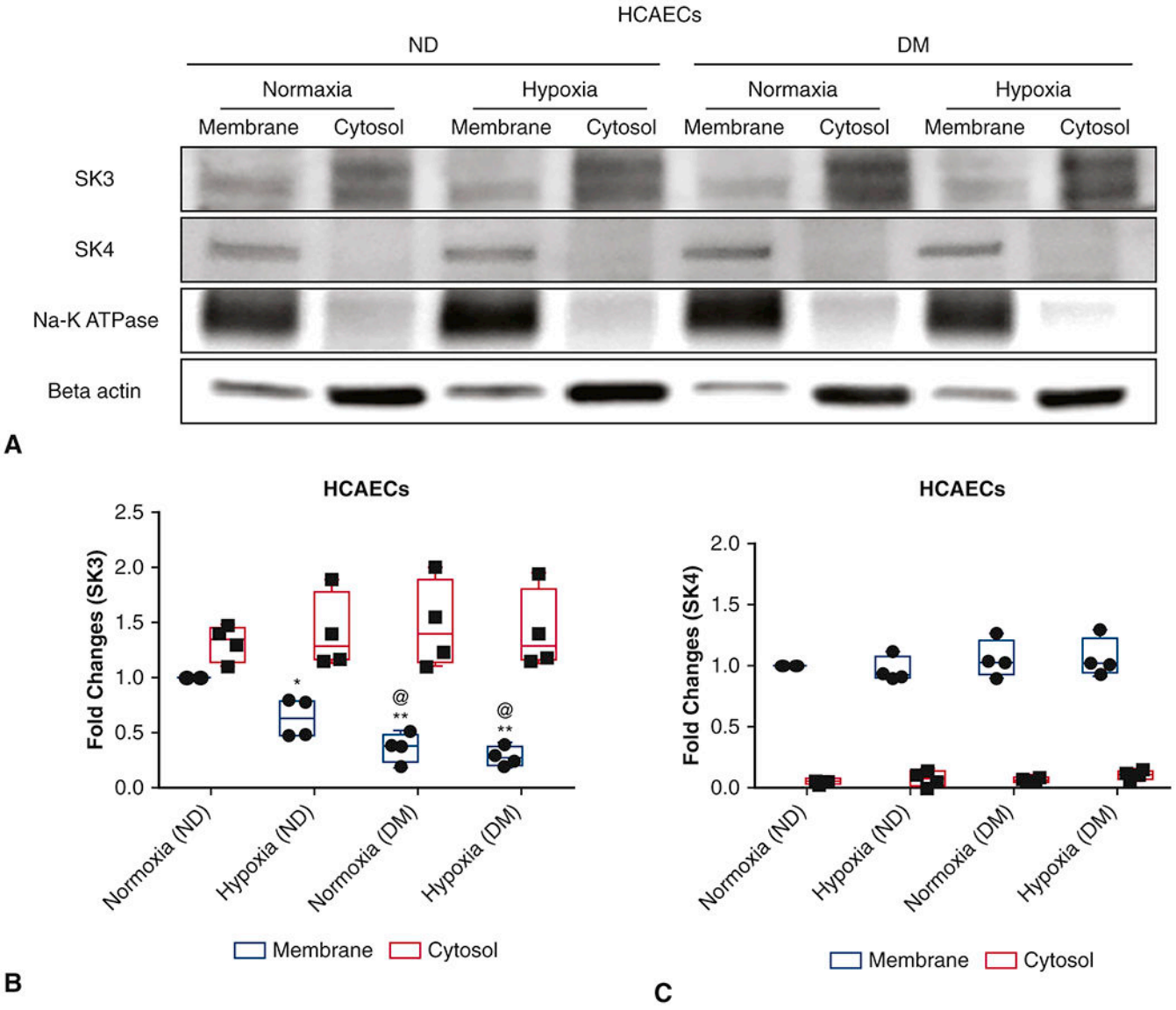

FIGURE 5.

A, Subcellular protein expression of small conductance calcium-activated potassium channels (SK) 3 and SK4 in human coronary arterial endothelial cell (*HCAEC*) membrane and cytosolic lysates in the setting of normoxia or 60-minute hypoxia with 60 minutes of reoxygenation. Hypoxia represents hypoxia/reoxygenation in the diabetic (*DM*) and nondiabetic (*ND*) groups. B and C, Densitometric evaluation of immunoblot band intensity of SK3 and SK4. **P* < .05 vs normoxia (ND); ***P* < .001 vs normoxia (ND); @*P* < .05 vs hypoxia (ND). Data are mean ± SD. n = 4 per group.

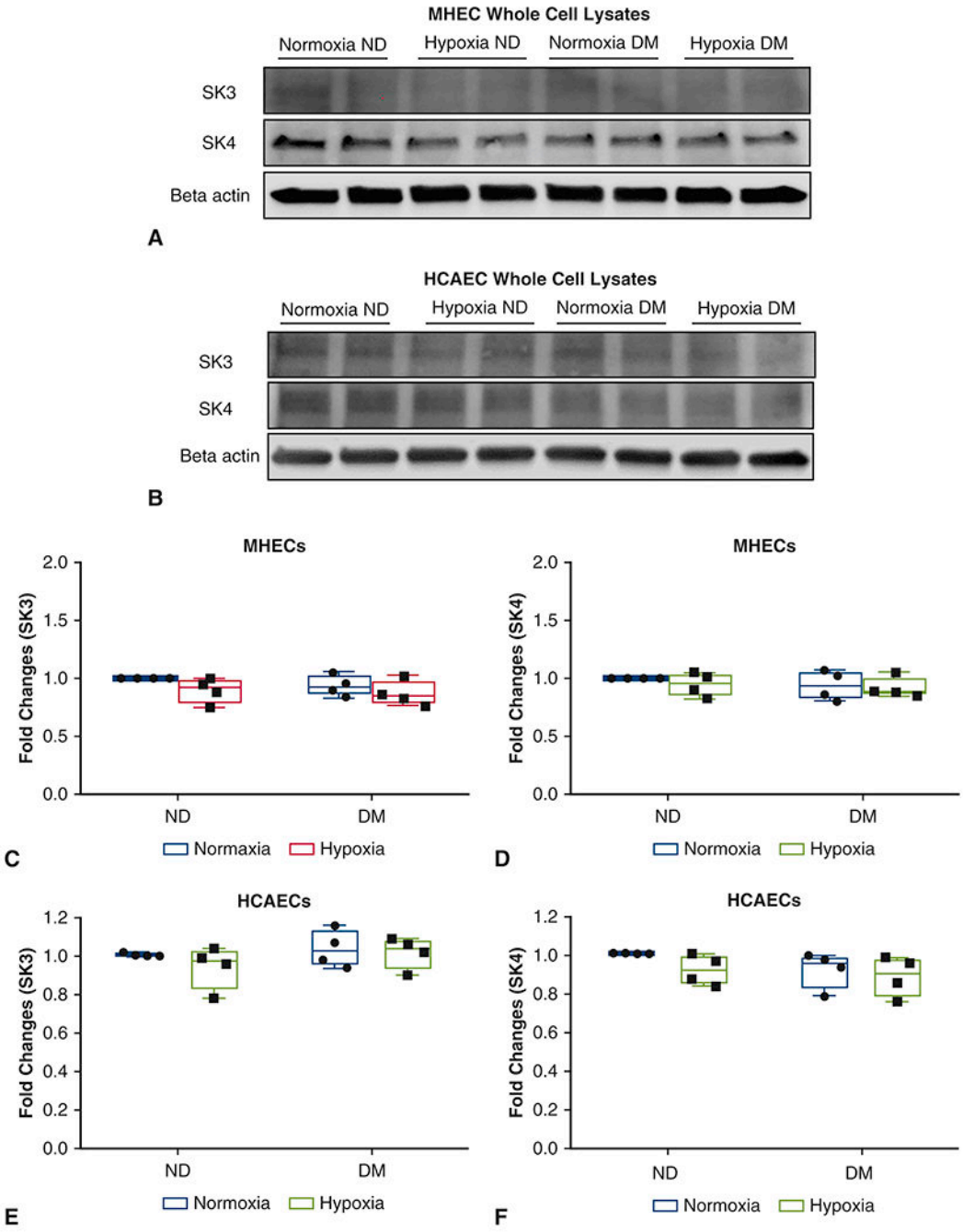

FIGURE 6.

A and B, Densitometric evaluation of immunoblot band intensity of small conductance calcium-activated potassium channel (*SK*) 3 and SK4 in mouse heart endothelial cells (*MHECs*) (A) and human coronary artery endothelial cells (*HCAECs*) (B); C and D, densitometric evaluation of immunoblot band intensity of SK3 (C) and SK4 (D) in the MHECs; E and F, densitometric evaluation of immunoblot band intensity of SK3 (E) and SK4 (F) in the HCAECs, data are mean ± SD. n = 4/group. ND, Nondiabetic; *DM*, diabetic.

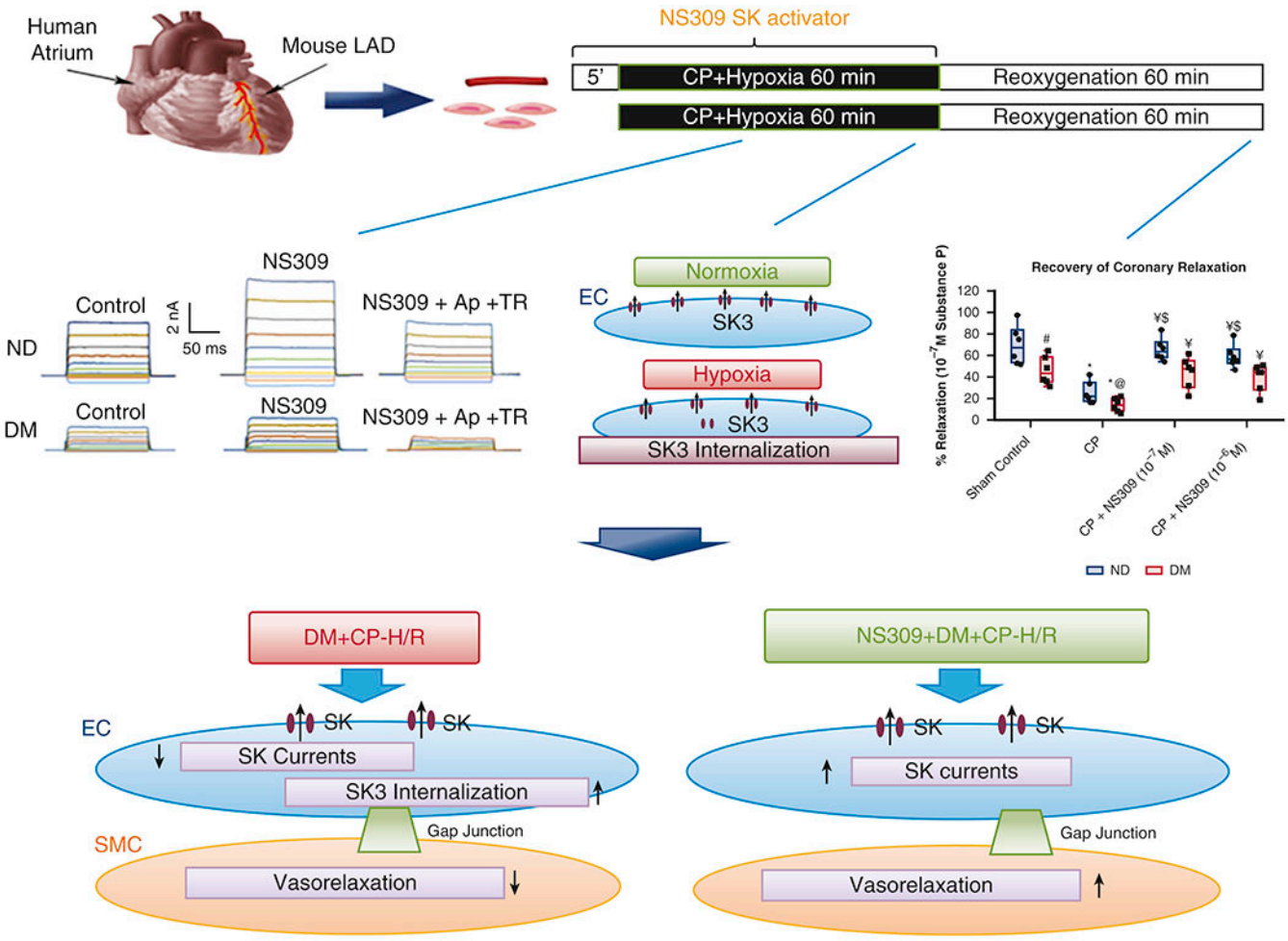

FIGURE 7.

Research summary showing that inclusion of the selective small conductance calcium-activated potassium channels (*SK*) channel activator NS309 in the cardioplegic solution increases endothelial SK currents, leading to protection of coronary endothelial function and coronary relaxation in the setting of diabetes (*DM*) and cardioplegic hypoxia and reoxygenation (*CP-H/R*). *LAD*, Left anterior descending artery; *ND*, nondiabetic; *NS309 + AP + TR*, NS309 plus apamin plus TRAM34; *EC*, endothelial cells; *SMC*, smooth muscle cells; NS309 + DM + CP-H/R, NS309 pretreatment plus NS309-enriched cardioplegia in the setting of diabetes plus cardioplegic hypoxia and reoxygenation.

TABLE 1.

Patient characteristics

Characteristic	ND	DM	*P* value
Age, y, mean ± SD	70 ± 7.6	72 ± 6.5	.67
Males/females, n	6/2	7/1	.52
HbAlc, %, mean ± SD	5.6 ± 0.39	8.38 ± 0.41	.0001
Preoperative blood glucose, mg/dL, mean ± SD	112 ± 14.7	152 ± 21	.008
Hypertension, n	4	5	.61
Obesity (BMI >30), n	2	3	.54
Hypercholesterolemia, n	3	4	.61
Atrial fibrillation, n	0	0	1.00
CABG only, n	2	5	.13
Valve replacement (n)	4	1	.11
CABG + valve replacement, n	2	2	1.00
Preoperative aspirin, n	3	5	.32
Preoperative β-blocker, n	2	4	.30
Preoperative ACEI, n	2	5	.13
Preoperative statins, n	4	7	.10
Antidiabetic drugs, n	0	8	.0001

ND, Nondiabetic; *DM*, diabetes mellitus; *SD*, standard deviation; *HbA1c*, hemoglobin A1c; *BMI*, body mass index; *CABG*, coronary artery bypass grafting; *ACEI*, angiotensin-converting enzyme inhibitor.