**Cardiovascular Consequences of Obesity and Type 2 Diabetes**

Vasonatrin peptide attenuates myocardial ischemia-reperfusion injury in diabetic rats and underlying mechanisms

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1Experiment Teaching Center, Fourth Military Medical University, Xi’an, China; 2Department of Physiology, Fourth Military Medical University, Xi’an, China; 3Department of Cardiology, Tangdu Hospital, Fourth Military Medical University, Xi’an, China; and 4Experimental Center, The Second Affiliated Hospital, School of Medicine, Xi’an Medical University, Xi’an, China

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**Shi Z, Fu F, Yu L, Xing W, Su F, Liang X, Tie R, Ji L, Zhu M, Yu J, Zhang H. Vasonatrin peptide attenuates myocardial ischemia-reperfusion injury in diabetic rats and underlying mechanisms. Am J Physiol Heart Circ Physiol 308: H281–H290, 2015. First published December 5, 2014; doi:10.1152/ajpheart.00666.2014.—Diabetes mellitus increases morbidity/mortality of ischemic heart disease. Although atrial natriuretic peptide and C-type natriuretic peptide reduce the myocardial ischemia-reperfusion damage in nondiabetic rats, whether vasonatrin peptide (VNP), the artificial synthetic chimera of atrial natriuretic peptide and C-type natriuretic peptide, confers cardioprotective effects against ischemia-reperfusion injury, especially in diabetic patients, is still unclear. This study was designed to investigate the effects of VNP on ischemia-reperfusion injury in diabetic rats and to further elucidate its mechanisms. The high-fat diet-fed streptozotocin-induced diabetic Sprague-Dawley rats were subjected to ischemia-reperfusion operation. VNP treatment (100 μg/kg iv, 10 min before reperfusion) significantly improved the instantaneous first derivation of left ventricle pressure (±LV dp/dtmax) and LV systolic pressure and reduced LV end-diastolic pressure, apoptosis index, caspase-3 activity, plasma creatine kinase (CK), and lactate dehydrogenase (LDH) activities. Moreover, VNP inhibited endoplasmic reticulum (ER) stress by suppressing glucose-regulated protein 78 (GRP78) and C/EBP homologous protein (CHOP). These effects were mimicked by 8-bromine-cyclic guanosine monophosphate (8-BrcGMP), a cGMP analog, whereas they were inhibited by KT-5823, the selective inhibitor of PKG. In addition, pretreatment with tauroursodeoxycholic acid (TUDCA), a specific inhibitor of ER stress, could not further promote the VNP’s cardioprotective effect in diabetic rats. In vitro H9c2 cardiomyocytes were subjected to hypoxia/reoxygenation and incubated with or without VNP (10−9 mol/l). Gene knockdown of PKG1α with siRNA blunted VNP inhibition of ER stress and apoptosis, while overexpression of PKG1α resulted in significant decreased ER stress and apoptosis. VNP protects the diabetic heart against ischemia-reperfusion injury by inhibiting ER stress via the cGMP-PKG signaling pathway. These results suggest that VNP may have potential therapeutic value for the diabetic patients with ischemic heart disease.**

**Diabetes mellitus (DM) is closely related to cardiovascular morbidity and mortality. People with DM have a risk of ischemic heart disease (IHD) two to five times greater than that in nondiabetic individuals (34). Importantly, diabetic patients have more severe and fatal myocardial infarctions than nondiabetic patients (15, 44). Identifying the cellular and molecular basis linking diabetes with increased IHD morbidity and mortality is not only scientifically important but may reveal new therapeutic targets against heart diseases in diabetes.**

Recent animal and human studies have revealed that various factors interfere with endoplasmic reticulum (ER) function and lead to accumulation of unfolded proteins, including ischemia-reperfusion (I/R), disturbance of calcium homeostasis, and overexpression of normal and/or incorrectly folded proteins (2, 23). Considerable evidence indicates that ER stress plays an essential role in cardiomyocyte death induced by myocardial ischemia-reperfusion (MI/R) (35, 39). Furthermore, the diabetic state enhances ER stress in cardiovascular complications such as IHD (9). Improved therapeutic intervention on ER stress may attenuate injury endured in myocardial ischemic diseases.

Natriuretic peptides (NPs) are a family of structurally related but genetically distinct peptides including atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP), which principally mediate the natriuretic, diuretic, vasorelaxant, and antiinflammatory effects via the NP-cGMP-PKG signaling pathway (25–27). It is elucidated that NPs reduce the MI/R damage in normal rats (3, 12). However, their effects on IHD in the diabetic state has rarely been reported before. Vasonatrin peptide (VNP) is an artificial synthetic chimera of ANP and CNP, which has proven to be more potentially diuretic, natriuretic, and vasorelaxant compared with other NPs, such as ANP and CNP (37). Our previous studies have demonstrated that VNP induced a dose-dependent vasorelaxation via the natriuretic peptide receptor (NPR) A/B-cGMP-BKCa signaling pathway (43). However, the effects of VNP on acute MI/R injury, especially in patients with diabetes, were still unclear.

Therefore, the aim of the present study was to 1) investigate the potential for exogenous VNP treatment to attenuate MI/R injury in diabetic rats; 2) determine whether inhibition of the ER stress is involved in VNP’s therapeutic effect; and 3) elucidate the underlying mechanisms with a special focus on the VNP-cGMP-PKG signaling pathway.
MATERIALS AND METHODS

High-fat diet-fed streptozotocin rat model. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health, NIH Publication No. 85-23, revised 1996 and were approved by the Fourth Military Medical University Committee on Animal Care. The high-fat diet-fed streptozotocin (HFD-STZ) rat model was developed as described previously with slight modifications (36). Normal 6-wk-old male Sprague-Dawley rats were fed with an HFD containing 40% fat (as a percentage of total kcal), 41% carbohydrate, and 18% protein or a normal chow diet consisting of 12% fat, 60% carbohydrate, and 28% protein for 4 wk. STZ (25 mg/kg ip; Sigma, St. Louis, MO) was injected once intraperitoneally to the rats after 4 wk of HFD feeding. HFD was continuously administrated after STZ injection, and then rats with a fasting plasma glucose level of >11.1 mmol/l 72-h post-STZ injection were considered diabetic and were studied. The normal diet-fed rats were used as nondiabetic controls. Glucose tolerance of various groups was further estimated by oral glucose tolerance test (OGTT) and intraperitoneal glucose tolerance test (IPGTT). Glucose (2 g/kg) was administered to 12-h-fasted rats either by gastric lavage or intraperitoneally, and blood samples were collected from the tail vein at 0 (before glucose load), 30, 60, 90, and 120 min for measurement of plasma glucose.

Myocardial infarction protocol. Rats were anesthetized by 3% pentobarbital sodium (30 mg/kg ip). Anesthesia was then maintained by 100 mg/kg sodium pentobarbital (100 mg/kg ip) and euthanized by decapitation.

Experimental groups. Nondiabetic rats were randomly assigned to two experiment groups (n = 8/group): Con + Sham, sham operation (rats underwent the same operation, and the ligature was looped through the myocardium next to the LAD) and receiving no treatment; and Con + MI/R (vehicle), MI/R operation and infusion of saline (0.5 ml iv, 10 min before reperfusion). Diabetic rats were randomized to one of the following groups (n = 8/group): DM + Sham; DM + MI/R + saline; and DM + MI/R + VNP (100 μg/kg, intravenous infusion at 25 μg·kg⁻¹·h⁻¹ for 4 h, 10 min before reperfusion). We tested the effects of VNP at 2.5, 25, and 250 μg·kg⁻¹·h⁻¹ on plasma cGMP level and arterial blood pressure in the preliminary study. It was found that 25 μg·kg⁻¹·h⁻¹ VNP induced an increase in plasma cGMP level without significant effect on mean arterial pressure, and this dose was used in this study; DM + MI/R + 8-bromine-cyclic guanosine monophosphate (8-Br-cGMP; 1 mg/kg ip, 20 min before reperfusion); DM + MI/R + VNP + KT-5823 (0.5 mg/kg ip, 20 min before reperfusion); and DM + MI/R + tauroursodeoxycholic acid (TUDCA) (50 mg/kg ip, 10 min before reperfusion).

Determination of apoptosis and myocardial infarction. At the end of the 4-h reperfusion, myocardial apoptosis was analyzed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay as described previously (33). Cardiac caspase-3 activity was measured by using caspase colorimetric assay kits (Chemicon International, Temecula, CA), as described in the previous study (17). At the end of the 6-h reperfusion, myocardial infarction was determined by means of a double-staining technique (33). Evans blue-stained areas [area not at risk (AAR)], triphenyltetrazolium chloride (TTC) stained areas (red staining, ischemic but viable tissue), and TTC-stained areas (red staining, ischemic and necrotic tissue) were quantified areas (% of the total heart area) and compared between groups.

Fig. 1. Diabetic rats subjected to myocardial ischemia-reperfusion showed significant impaired cardiac functions and increased myocardial injury and enhanced endoplasmic reticulum (ER) stress. Diabetes mellitus (DM) + myocardial ischemia-reperfusion (MI/R) group showed decreased the instantaneous first derivation of left ventricle pressure (±LV dP/dtmax) compared with the Con + MI/R group in A and B. There was decreased left ventricle developed pressure (LVDP) in DM + I/R hearts compared with Con + MI/R hearts (C). No significant differences in left ventricle end-diastolic pressure (LVEDP) were observed between these 2 groups (D). Significant increased infarct size (E: myocardial infarct size expressed as percentage of area-at-risk (IF/AAAR)), apoptosis index (F), caspase-3 activity (G), and plasma creatine kinase (CK) and lactate dehydrogenase (LDH) activities (H) were found in the DM + MI/R group compared with the Con + MI/R group. Moreover, increased C/EBP homologous protein (CHOP; J) and glucose-regulated protein 78 (GRP78; K) expression were induced by ischemia-reperfusion in diabetic hearts compared with nondiabetic hearts. Values presented are means ± SE; n = 8/group. Differences were compared by ANOVA followed by Bonferroni correction for post hoc t-test where appropriate. ∗P < 0.05, ∗∗P < 0.01 vs. Con + Sham; #P < 0.05, ##P < 0.01 vs. Con + MI/R; ΔΔP < 0.01 vs. DM + Sham.
staining negative areas (infarcted myocardium) in each slice were determined by planimetry on a computer with Sigma Scan software. The myocaridal infarct size was expressed as a percentage of infarct area (INF) over total myocardial infarct size expressed as percentage of AAR (INF/AAR × 100%).

Biochemical estimation. After the reperfusion of 4 h, blood samples were obtained from abdominal aorta and then centrifugated at 3,000 g for 10 min to obtain plasma for LDH, creatine kinase (CK), malondialdehyde (MDA), and SOD measurement. The procedures strictly followed manufacturer’s instructions. All chemicals used for biochemical analysis were obtained from Nanjing Jiangcheng Bioengineering Institute (Nanjing, China).

Cell culture and treatments. H9c2 cardiomyocytes were purchased from the Cell Bank of Chinese Academy of Science (Beijing, China). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO) supplemented with 10% inactivated fetal bovine serum (FBS; GIBCO), 500 μg/ml penicillin, and 500 μg/ml streptomycin (GIBCO) at 37° in a humidified atmosphere with 5% CO2. As suggested by some laboratories, concentrations of glucose approaching 10 mM are prediabetic levels, and concentrations of glucose above 10 mM are analogous to a diabetic condition within the cell culture system. Accordingly, DMEM containing 25-mM levels of glucose has been applied in several studies to explore the pathophysiology of diabetes as well as to test agents for the treatment of diabetic complications, in which DMEM with 5.5 mmol/l glucose was used to culture H9c2 cells as a control (4, 11, 20). In this study the cells were incubated with control (5.5 mmol/l) or high-glucose/high-fat (HG/HF) medium containing glucose (25 mmol/l) and saturated FFA palmitate (16:0; 500 μmol/l) as previously described (41). After 18 h of HG/HF incubation, cardiomyocytes were then exposed to hypoxia/reoxygenation (H/R) treatment as previously described (40). In brief, H9c2 cardiomyocytes were exposed to glucose-free serum-free culture medium and transferred into a Modular Incubator Chamber (Billumps-Rothenberg) flushed with 5% CO2–95% N2 for 3 h of hypoxia. Then, the culture medium was replaced with fresh oxygenated normal or VNP (10⁻⁸ mol/l) cultured medium, and the dishes were transferred to a normoxic incubator (95% air–5% CO2) for 6 h of reoxygenation. We tested the effects of VNP at 10⁻¹⁰, 10⁻⁹, 10⁻⁸, 10⁻⁷, and 10⁻⁶ mol/l on intracellular cGMP concentrations in H9c2 cells in vitro. It was found that VNP increased the cGMP level in a concentration-dependent manner in vitro. The cGMP level at the concentration of 10⁻⁸ mol/l VNP is 130 ± 9 nmol/l, which is similar to the plasma cGMP level at the dose of 25 μg·kg⁻¹·h⁻¹ VNP treatment in vivo. Therefore, the VNP concentration of 10⁻⁸ mol/l was employed in vitro in our study. The number of viable H9c2 cells was measured after 48 h by 3-[4,5-yl]-2,5-diphenylylterazolium bromide (MTT) cell viability assay.

Adenovirus infection. Adenoviral vectors for overexpression of PKG1α were used as previously described (38). Viruses were propagated in 293 cells and kept at −80°C with a titer of 10¹¹⁻¹² plaque-forming units per milliliter before using. Gene transfer with adenovirus encoding GFP was used as an internal control. Cardiomyocytes plated at a density of 0.5 to 1 × 10⁶/cm² were infected with adenovirus at 50 multiplicity of infection for 2 h at 37°C in a humidified, 5% CO2 incubator. Subsequently, the cells were cultured in serum-free DMEM media for an additional 24 h before processing.

Transfection of siRNAs. Double-stranded RNAi for selective silencing of PKG1α with a final concentration of 100 nM was transfected into cells (FuGENE Reagent; Roche Applied Science, Mannheim, Germany) according to the manufacturer’s instructions. After transfection, cells were performed the same H/R treatment as described above. Scrambled RNAi was used as control. Knockdown of gene expression was identified by Western blot analysis 48 h after transfection.

**Fig. 2.** Vasonatin peptide (VNP) exerted cardioprotective effects against MI/R damage in both nondiabetic and diabetic rats. MI/R-stimulated GRP78 and CHOP was decreased with VNP treatment in A and B. VNP treatment increased ±LV dP/dt max in C and D and reduced plasma CK and LDH activities in E and F in both nondiabetic and diabetic animals subjected to MI/R. Compared with DM + MI/R group, VNP treatment exerted an ant apoptotic effect on diabetic hearts as evidenced by the decreased myocardial apoptotic index and caspase-3 activity in G and H. G, top, representative photomicrographs of in situ detection of apoptotic myocytes by terminal deoxynucleotidiyl transferase dUTP nick end labeling (TUNEL) staining. Green fluorescence shows TUNEL-positive nuclei; blue fluorescence shows nuclei of total cardiomyocytes; red fluorescence shows cardiomyocytes; original magnification: ×400. G, bottom: percent age of TUNEL-positive nuclei in heart tissue sections. Values presented are means ± SE; n = 8/group. Statistical significances of the differences were determined by Bonferroni correction for post hoc t-test or Student’s t-test where appropriate. **P < 0.01 vs. Sham; #P < 0.05, vs. MI/R; *P < 0.05, **P < 0.01 vs. DM + MI/R.
Western blotting. Samples were lysed with lysis buffer. After sonication, the lysates were centrifuged, and proteins were separated by electrophoresis on SDS-PAGE and then transferred onto PVDF-Plus membrane (Micron Separations, Westborough, MA). After being blocked with 5% milk, the immunoblots were probed with anti-glucose-regulated protein 78 (anti-GRP78), C/EBP homologous protein (CHOP), PKG1α, Akt, pAkt (Ser 473), ERK1/2, pERK1/2 (Thr202/Tyr204), and β-actin (Cell Signaling, Beverly, MA) overnight at 4°C followed by incubation with the corresponding secondary antibodies at room temperature for 1 h. The blots were visualized with ECL-plus reagent.

Statistical analysis. All values are presented as means ± SE. Differences were compared by ANOVA followed by Bonferroni correction for post hoc t-test where appropriate; * P < 0.05 was considered to be statistically significant. All statistical tests were performed with the GraphPad Prism software v.5.0 (GraphPad Software, San Diego, CA).

RESULTS

Characterization of animals. The HFD-STZ-induced diabetic rats with fasting plasma glucose level of above 11.1 mmol/l 72 h post-STZ injection were considered have developed diabetes (32). To confirm that diabetic model was successfully developed, IPGTT and OGTT were performed. Compared with the normal control, HFD-STZ rats showed significantly increased blood glucose and impaired IPGTT and OGTT, indicating that the type 2 diabetic model was successfully developed in the present study (Supplemental Material for this article is available online at the Journal website; Supplemental Fig. S2; n = 10; all P < 0.05).

Aggravation of cardiac dysfunction and injury and ER stress in diabetic rats subjected to MI/R. It has been reported that diabetes enhances susceptibility to MI/R injury in many previous studies (1). As shown in Fig. 1, A and B, there was no significant difference between control sham and DM sham animals in the instantaneous first derivation of left ventricle pressure (±LV dP/dtmax). However, diabetic rats (DM + MI/R group) showed more severe cardiac functional impairment induced by MI/R than the control group (Con + MI/R) as evidenced by reduced ± LV dP/dtmax at R 4 h (n = 8; P < 0.05). There was decreased developed pressure in DM + I/R hearts compared with Con + MI/R hearts (Fig. 1C). No significant differences in end-diastolic pressure were observed between these two groups (Fig. 1D). Similarly, diabetic rats showed larger INF and increased myocardial apoptotic index, caspase-3 activity, and plasma CK and LDH activities (Fig. 1, E, F, G, and H; all P < 0.05 vs. Con + MI/R), indicating that that diabetic state aggravates cardiac injury caused by MI/R. In addition, it is shown in Fig. 1, I and J, that diabetes alone and I/R alone can increase GRP78 and CHOP (2 well-known ER stress markers) expression compared with control sham, while significant aggravated ER stress was induced by I/R in diabetic hearts compared with nondiabetic hearts.

Fig. 3. VNP but not atrial natriuretic peptide (ANP) or C-type natriuretic peptide (CNP) attenuated MI/R injury in diabetic rats. VNP treatment reduced infarct size (A) and lowered plasma CK (B) and LDH activities (C) and decreased caspase-3 activity (D) compared with DM group. No significant differences in these parameters were observed when treated with ANP or CNP at the same dose in diabetic rats compared with DM group. Values presented are means ± SE; n = 8/group. # P < 0.05, ##P < 0.01 vs. DM; #P < 0.05, ###P < 0.01 vs. DM + VNP.

H284 VNP ATTENUATES MI/R INJURY IN DIABETES AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00666.2014 • www.ajpheart.org

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Exogenous VNP treatment improved cardiac function and inhibited apoptosis in diabetic hearts. To examine whether VNP treatment improves cardiac function in nondiabetic and diabetic animals, VNP was infused through external jugular vein 10 min before reperfusion (25 μg·kg⁻¹·h⁻¹ for 4 h). MI/R-stimulated GRP78 and CHOP was decreased with VNP treatment (Fig. 2, A and B). VNP treatment increased ±LV dP/dt max (Fig. 2, C and D) and reduced plasma CK and LDH activities (Fig. 2, E and F) in both nondiabetic and diabetic animals subjected to MI/R. These results suggested that VNP could further protect the heart in diabetes in addition to the baseline cardioprotective effects. Then, we observed whether VNP inhibits cardiomyocyte apoptosis. As shown in Fig. 2, G and H, VNP treatment exerted antiapoptotic effects as evidenced by the decreased myocardial apoptotic index and caspase-3 activity. However, ANP or CNP treatment did not exert cardioprotective effects on infarct size, plasma CK and LDH activities, and caspase-3 activity in diabetic MI/R rats (Fig. 3). Together with the cardiac function results, these data indicated that VNP treatment exerted the antiapoptotic effect, thus improving the cardiac function in diabetic rats subject to MI/R.

Exogenous VNP treatment reduced ER stress and apoptosis in diabetic MI/R hearts via the cGMP-PKG signaling pathway. To further elucidate the mechanism underlying VNP’s cardio-

Fig. 4. VNP inhibited ER stress and apoptosis in diabetic MI/R hearts through the cGMP-PKG signaling pathway. MI/R diabetic heart showed significant decreased PKG expression, while VNP significantly upregulated it in A. VNP markedly decreased MI/R-stimulated GRP78 and CHOP expressions, which was fully mimicked by 8-bromine-cyclic guanosinemonophosphate (8-Br-cGMP) and inhibited by KT-5823 in B and C. The VNP group showed significantly reduced myocardial apoptotic index and caspase-3 activity, which was also mimicked by 8-Br-cGMP and inhibited by KT-5823 in D and E. 8-Br-cGMP, 8-bromine-cyclic guanosinemonophosphate; KT-5823, (8R,9S,11S)-(-)-(2)-2-methyl-9-methoxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo(a,g)cyclocta9(cde)trinen-1-one. Values presented are means ± SE; n = 8/group. Differences were compared by ANOVA followed by Bonferroni correction for post hoc t-test where appropriate. *P < 0.05, **P < 0.01 vs. Sham; #P < 0.05, ##P < 0.01 vs. MI/R; ΔP < 0.05, ΔΔP < 0.01 vs. VNP.
protective effects, we determined the expression of GRP78 and CHOP in diabetic hearts. As seen in Fig. 4, B and C, compared with sham-operated hearts, the MI/R diabetic myocardium showed a significant increased expression in GRP78 and CHOP, whereas VNP treatment markedly decreased MI/R-stimulated GRP78 and CHOP ($P < 0.05$ vs. MI/R). These results suggested that VNP treatment inhibits ER stress in diabetic MI/R hearts. Moreover, we found a significant decrease in PKG level in MI/R diabetic hearts, which was upregulated by VNP treatment (Fig. 4A; both $P < 0.05$). Then, 8-Br-cGMP, a cGMP analog, and KT-5823, the selective inhibitor of PKG, were used to further elucidate the mechanisms. VNP-induced ER stress inhibition was fully mimicked by 8-Br-cGMP and was blunted by inhibition of PKG with KT-5823 (Fig. 4, B and C). Similarly, 8-Br-cGMP decreased the apoptotic index and caspase-3 activity, which were blunted by KT-5823 (Fig. 4, D and E). All these data demonstrated that VNP inhibited ER stress and apoptosis through VNP-cGMP-PKG signaling pathway.

VNP inhibited ER stress and protected cardiomyocytes from H/R-induced apoptosis in vitro. Our in vivo experimental results demonstrated that VNP treatment significantly attenuated MI/R injury by inhibiting ER stress. In addition, the VNP-cGMP-PKG signaling pathway was involved its therapeutic effect. To support a causative link between the VNP-cGMP-PKG signaling pathway and VNP-attenuated ER stress and myocardial injury, we employed H9c2 cardiomyocytes to obtain more evidence. As illustrated in Fig. 5A, the lower level of PKG was found in H9c2 cardiomyocytes exposed to H/R, which was upregulated by VNP treatment. The cells’ death as measured by MTT and TUNEL assay was decreased significantly by the treatment with VNP (Fig. 5, B and C). Furthermore, VNP downregulated GRP78 and CHOP levels in the cardiomyocytes exposed to H/R, indicating that VNP treatment inhibits ER stress (Fig. 5, D and E). These data suggest that VNP attenuates H/R-induced apoptosis via the regulation of VNP-cGMP-PKG signaling in vitro (all $P < 0.05$).

**PKG1α contributed to VNP’s protective effect in H9c2 cells subjected to H/R injury.** To directly clarify the critical role of VNP-cGMP-PKG1 signaling in VNP-attenuated H/R injury, we investigated the effects of upregulating or downregulating PKG1α expression on VNP’s effects in H9c2 cell. As expected, VNP reduced ER stress and apoptosis in scrambled RNAi-treated cells, which were significantly blunted in PKG1α siRNA-treated cells. On the other hand, overexpression of PKG1α resulted in significantly decreased cellular levels of the ER stress marker and cell apoptosis following H/R with or without VNP (Fig. 6). All these data demonstrated that VNP-cGMP-PKG1 signaling contributes to VNP’s inhibition of ER stress and cell injury in H/R H9c2 cells (all $P < 0.05$).

Fig. 5. VNP inhibits hypoxia/reoxygenation (H/R)-induced H9c2 cardiomyocytes ER stress and apoptosis. VNP upregulated the PKG expression in H9c2 cells, which was suppressed by H/R treatment in A. 3-[4,5-yl]-2,5-diphenyltetrazolium bromide (MTT) and TUNEL assay results showed that VNP treatment rescued cell death in B and C. VNP downregulated GRP78 and CHOP expression in the cardiomyocytes exposed to H/R in D and E. Values presented are means ± SE; $n = 8$ / group. Differences were compared by ANOVA followed by Bonferroni correction for post hoc t-test where appropriate. *$P < 0.05$, **$P < 0.01$ vs. Normal; $\#P < 0.05$, $\##P < 0.01$ vs. H/R.
In vivo study confirmed that VNP confers cardioprotective effects by inhibiting ER stress. Having recognized that VNP inhibited ER stress and cell injury via VNP-cGMP-PKG signaling in the in vitro study, we further confirmed the molecular signaling mechanisms in the diabetic rats in vivo. As expected, GRP78 and CHOP were both significantly downregulated in the diabetic heart subjected to MI/R injury either by intravenous infusion of VNP or TUDCA, a specific inhibitor of ER stress (Fig. 7, A and B; vs. MI/R). Furthermore, Akt and ERK were significantly upregulated by both VNP and TUDCA treatment in diabetic hearts (Fig. 7, C and D; vs. MI/R), suggesting that inhibition of ER stress activated Akt and ERK signaling. As shown in Fig. 7, E, F, and G, both VNP and TUDCA groups showed significant smaller infarct size and lower apoptotic index and caspase-3 activity (all \( P < 0.05 \)). We further measured the accumulated amount of MDA and SOD released during reperfusion after ischemia (Fig. 7, H and I). Both VNP and TUDCA significantly decreased MDA and increased SOD activity, which demonstrated that VNP and TUDCA treatment inhibited oxidative stress (all \( P < 0.05 \)).

These data all indicate that inhibition of ER stress was involved in VNP’s antiapoptotic effect in diabetic hearts subjected to MI/R injury. In addition, activation of Akt and ERK may be involved in this effect. The proposed cardioprotective signaling pathway by VNP was shown in Fig. 7J.

**DISCUSSION**

The major findings of this study are as follows. First, we demonstrated for the first time that exogenous VNP treatment significantly attenuates MI/R injury in diabetic rats. Second, we also demonstrated that VNP exerts cardioprotective effect by inhibiting ER stress. Third and most important, the VNP-cGMP-PKG1 signaling pathway is involved in VNP’s inhibition of ER stress both in vivo and in vitro. To the best of our knowledge, this is the first demonstration that exogenous VNP treatment confers cardioprotection against I/R injury in diabetic hearts by inhibiting the ER stress via the VNP-cGMP-PKG signaling pathway.

DM is a risk factor for the development of coronary artery disease and chronic heart failure and confers a major adverse impact on morbidity and mortality (22). It is known in previous studies that diabetes can impair responses of the myocardium to protective interventions. Protection afforded by preinfarct angina is lost in diabetic patients (16). In animal models, some pharmacological agents failed to exert their cardioprotection in diabetic hearts (10, 11). Thus further studies are needed to
seek novel strategies and targets to reduce MI/R-induced injury in diabetic state. To address this need, we developed the type 2 diabetes animal model based on a HFD and a single dose of STZ injection in the present study. HFD-elicited insulin resistance and administration of STZ decreased insulin levels, such that the insulin-resistant rats were unable to maintain normal glucose levels and develop hyperglycemia. Significantly increased blood glucose level and impaired IPGTT and OGTT were found in diabetic animals, thereby making this model a good representation of the human type 2 diabetic condition. After developing this HFD-STZ-induced diabetic model, we observed that the diabetic state aggravated MI/R injury and showed more severe myocardial functional impairment than normal state, which was consistent with the previous study (5, 41).

NPs are a family of structurally related, but genetically distinct, polypeptide hormones that regulate mammalian blood volume and blood pressure (19). The ability of the NPs to modulate cardiac function and cell proliferation has already been recognized. Exogenous BNP exerts a direct antihypertrophic action in both neonatal and adult rat cardiomyocytes (28). Meanwhile, ANP and CNP reduce the MI/R damage in normal rats (3, 12). As a synthetic NP, VNP has been reported to have similar, yet different, cardiovascular bioactivity compared with its “parents” ANP and CNP. Wei et al. (37) proved VNP to be more potential than ANP or CNP in arterial and venous vasodilation, which may further reduce preload and postload of the heart and promote the functional recovery after MI/R. Moreover, as VNP is a synthesized peptide that is not found in nature, it may be more slowly degraded by neutral endopeptidase 24.11, which results in a more prolonged half-life than in the endogenous NPs (21). Taken together, the above properties of VNP suggest that the cardioprotection of VNP might be superior to that of ANP or CNP, and it was shown in our study that VNP exerts its cardioprotective effects against MI/R injury in the diabetic state, in which VNP and CNP have failed to do so. To the best of our knowledge, this is the first demonstration of its effect on MI/R in the diabetic animal.

Of the many theories regarding the development of MI/R injury in diabetic state, the enhanced ER stress during the acute reperfusion phase is an appealing one that is supported by a large foundation of experimental evidence (9, 35, 39). ER stress is initially increased in response to I/R in an attempt to restore normal function, but prolonged or severe ER stress can lead to apoptotic cell death and impaired cardiac function (24). Moreover, recent studies have also shown that the ER stress-associated metabolic disturbances altered Ca\(^{2+}\) handling, oxidative stress, and apoptosis and participated in the development of diabetes (42). In the present study, we found that the expression of GRP78 and CHOP, which have been identified as important ER stress markers, was increased in diabetic hearts subjected to MI/R. The expressions of GRP78 and CHOP were suppressed in the VNP group, which indicated that VNP inhibited ER stress and thus reduced cardiomyocyte apoptosis in diabetic hearts subjected to MI/R injury. We further evaluated oxidative stress by measuring serum MDA and SOD levels and found that VNP significantly inhibited oxidative stress induced by MI/R. Previous studies have shown that removal of reactive oxygen
species by the antioxidant N-acetyl-L-cysteine significantly attenuated aldosterone-induced activation of ER stress and apoptosis (7). Together, these data suggest that VNP may suppress ER stress by inhibiting oxidative stress in the diabetic heart subject to MI/R injury.

Previous studies have confirmed that most functions of NPs are mediated through the elevation of intracellular cGMP after their binding to NPRs, NPR-A and NPR-B, which are coupled to the particulate guanylyl cyclase (8). Specifically, ANP and BNP mainly bind to NPR-A, while CNP functions via NPR-B. A recent study has shown that cGMP stimulated by VNP in NPR-B-transfected cells was 11-fold higher than that of NPR-A (18). It seems that VNP binds preferentially to NPR-B and stimulates cGMP production. cGMP positively regulates PKG but inhibits/activates phosphodiesterases (PDEs), which are predominant in the cardiovascular system (13, 31). Importantly, we here observed that diabetic rats exhibited decreased myocardial PKG expression while VNP upregulated it. Numerous studies have reported that PKG signaling plays a key role in cardioprotection, especially in MI/R injury (29). Although some mechanisms have been elucidated, the relationship between ER stress and PKG signaling is still unknown. Here, we found that VNP-induced inhibition of ER stress was fully mimicked by 8-Br-cGMP, a cGMP analog, and was blunted by KT-5823, the selective inhibitor of PKG. Similar results were found in the apoptotic index and caspase-3 activity. All these data strongly suggested that VNP inhibited ER stress through the VNP-cGMP-PKG signaling pathway, which is confirmed by the H9c2 cardiomyocytes with gene transfer or knockdown of PKG-1α. It has been reported that PKG signaling plays a key role in cardioprotection, especially in MI/R injury. PKG-mediated blockage of mitochondrial permeability transition and cytochrome c release plays a key role in nitric oxide’s protection effect on the ischemic heart. In addition, PKG-dependent ERK phosphorylation is indispensable for the induction of endothelial and inducible nitric oxide synthase and Bcl-2 and the resulting cardioprotection by sildenafil, a selective inhibitor of PDE type 5, which exerts degradation of cGMP (6, 30). Here, we demonstrated for the first time that the VNP-cGMP-PKG1 signaling pathway mediates VNP’s inhibition of ER stress and apoptosis both in vivo and in vitro and inhibition of oxidative stress is involved in this effect.

Having achieved both in vivo and in vitro evidence of that VNP’s cardioprotective effect, we further confirm its underlying mechanisms in diabetic animal using TUDCA, a specific inhibitor of ER stress. It was found that TUDCA achieved similar cardioprotective effects as VNP by inhibiting ER stress but did not further enhance VNP’s effect. These experimental results further suggested that VNP exerts its cardioprotection through inhibition of ER stress.

It has been reported that ER stress may compromise cardiac contractile and intracellular Ca2+ properties, possibly through the Akt/GSK3β-dependent impairment of mitochondrial integrity, while activation of endogenous Akt/IAPs and MEK/ERK plays a critical role in controlling cell survival by resisting ER stress-induced cell death signaling (14, 46). In addition, Yuan et al. (45) demonstrated that ischemic postconditioning protects the brain from I/R injury by attenuating ER stress-induced apoptosis through the phosphatidylinositol 3-kinase-Akt pathway. Here, we found that myocardial Akt and ERK1/2 both were significantly increased in the VNP group and TUDCA group, suggesting that inhibition of ER stress may activate Akt and ERK signaling. The underlying mechanisms need to be further investigated.

In conclusion, VNP treatment protects against MI/R injury by inhibiting ER stress through cGMP-PKG signaling in diabetic rats (Fig. 7J). These data have provided important new insights into our understanding of VNP’s cardioprotective effects, highlighting the opportunity of a novel therapy for the diabetic patients with IHD.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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