Nicorandil, a $K_{\text{atp}}$ channel opener, alleviates chronic renal injury by targeting podocytes and macrophages

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Inhibition of the renin-angiotensin system (RAS) has been shown to be protective in most forms of chronic kidney disease (CKD). Nevertheless, blockade of RAS primarily slows renal disease, as opposed to arresting progression (21). Thus additional therapeutic strategies are needed.

Nicorandil, 2-[pyridin-3-ylcarbonyl]aminoethyl nitrate, is a clinically proven anti-anginal agent that causes vasodilatation by opening ATP-dependent K channels and also releasing nitric oxide (NO) (1). These pharmacological effects are associated with the reduction of oxidative stress (3).

Nicorandil has been recently reported to have benefit in experimental renal disease, including in the renal injury induced by ischemia-reperfusion (17) and glomerulonephritis (19) in the rats. In humans, the pharmacokinetics of nicorandil were examined in healthy volunteers and patients with impaired renal function (4, 10, 22). However, no studies have examined whether nicorandil may benefit CKD. Furthermore, the specific mechanisms by which nicorandil causes renoprotection are not known.

Here, we examined if nicorandil prevents the progression of CKD in the rat remnant kidney (RK) model.

METHODS

Experimental protocols. All animal experiments were performed in accordance with, and approved by, the Institute Animal Care and Use Committee of the University of Colorado, an AAALAC accredited independent review body within the University’s Office of Laboratory Resources. Male Sprague-Dawley rats (200–240 g) underwent baseline blood pressure (BP) and renal function assessments and were randomly assigned to the RK group or the sham-operated control group. For the RK group, a right subcapsular nephrectomy was performed and followed by surgical resection of the upper and lower one-thirds of the left kidney. Rats were divided into four subgroups: 1) sham group, 2) RK group, 3) RK with low dose nicorandil (3 mg·kg$^{-1}$·day$^{-1}$), and 4) RK with high dose nicorandil (30 mg·kg$^{-1}$·day$^{-1}$) ($n$ = 7 for each group). All rats were fed ad libitum. Two weeks after surgery, rats were randomized based on blood urea nitrogen (BUN) level before starting nicorandil treatment (Chugai Pharmaceutical, Tokyo, Japan). To constantly administer the same amount of nicorandil (30 mg·kg$^{-1}$·day$^{-1}$), the concentration of nicorandil in the drinking water was adjusted every 3 days, along with the water intake volume. Water bottles were monitored daily throughout the study to ensure that no leakage occurred. Systolic BP was measured at 0, 4, and 12 wk using a tail-cuff sphygmomanometer (Visitech BP-2000, Visitech Systems, Apex, NC). Urine was collected overnight using metabolic cages (Techniplast, Exton, PA) before death. All of the rats were killed at 12 wk to obtain blood samples and kidney tissues.

Laboratory studies. Urine albumin, urine 8-hydroxy-2’-deoxyguanosine (8-OHdG), xanthine oxidase activity, and urine creatinine were measured with Nephret (Exocell, Philadelphia, PA), OxiSelect Oxidative DNA Damage ELISA Kit (Cell Biosciences, San Diego, CA), xanthine oxidase assay kit (Cayman, Ann Arbor, MI), and Creatinine LiquiColor Test (enzymatic methodology; Stanbio, Boerne, TX), respectively. Uric acid concentration in the renal cortex was measured at 0, 4, and 12 wk using a tail-cuff sphygmomanometer (Visitech BP-2000, Visitech Systems, Apex, NC). Urine was collected overnight using metabolic cages (Techniplast, Exton, PA) before death. All of the rats were killed at 12 wk to obtain blood samples and kidney tissues.

Histological analysis. Formalin-fixed, paraffin-embedded sections (2.5 μm) were stained with the periodic acid-Schiff reagent for light microscopy. On coronal sections of the kidney, all glomeruli (50–100 glomeruli) were examined to evaluate glomerulosclerosis. Glomerulosclerosis was defined as obstruction of capillary lumen caused by
mesangial expansion or collapsed capillaries (15). Kidney sections were observed by two investigators in a blinded manner.

**Primary antibodies for immunohistochemistry and Western blotting.** Goat anti-human type IV collagen antibody (Southern Biotech, Birmingham, AL) and mouse anti-rat CD68 antibody (AbD Serotec, Oxford, UK) were used for glomerular injury. Rabbit anti-human WT-1 antibody (Santa Cruz, Santa Cruz, CA) and rabbit anti-human NPHS2 (podocin) antibody (Abcam, Cambridge, MA) were used to detect podocytes. To examine the tubulointerstitial injury, goat anti-human type III collagen antibody (Southern Biotech) was used. Mouse anti-rat CD11b/c equivalent antibody (OX42) (Abcam) was for identifying the phenotype of immune cell in the interstitium. Goat anti-human sulfonlurea receptor (SUR)-2B (C-15) antibody (Santa Cruz) and rabbit anti-human ABCC9 antibody (Abcam) were for detecting of SUR. Rabbit anti-rat manganese SOD (MnSOD) (Enzo Life Sciences, Farmingdale, NY), rabbit anti-human xanthine oxidase (H-110) antibody (Santa Cruz), mouse anti-nitrotyrosine antibody (Millipore, Billerica, MA), mouse anti-GAPDH antibody (Millipore), and rabbit anti-mouse heme oxygenase-1 antibody (Stressgen, Ann Arbor, MI) were also used.

**Immunohistochemistry.** Either formalin or methyl Carnoy’s solution-fixed, paraffin-embedded sections were used for immunohistochemistry, as previously described (6). To identify the cell type, serial section (1.5 μm) technique was used with specific markers of cell type. Briefly, after deparaffinization, the sections were treated with 3% H2O2 for 10 min to inactivate endogenous peroxidase activity. For WT-1, CD68, CD11b/c, and SUR2, the sections were treated with 10 mM citrate buffer (pH 6.0) for 30 min in a steamer for antigen retrieval. The sections were incubated with primary antibodies over-night at 4°C, followed by treatment with secondary antibodies for 30 min. Color development was achieved using diaminobenzidine with/without nickel chloride. All glomeruli at ×400 magnification or 15 fields (each field/0.4 mm²) in each section were used to count the number of positive cells for CD68, CD11b/c, or WT-1. To assess the type IV collagen and podocin positive area, the digital images at ×400 magnification were analyzed using Image scope software (Aperio Technologies, Vista, CA). The percent positive area was determined as the diaminobenzidine-positive pixel per total pixel in interest area from all glomeruli in each section. Likewise, positive area for type III collagen in interstitium was also determined as percent positive area with 15 fields (each field/0.4 mm²).

**Western blotting.** Kidney cortex was homogenized in cell lysis buffer (Cell Signaling, Danvers, MA) at 4°C. Briefly, samples were processed for SDS-PAGE, and electrotransferred onto a nitrocellulose membrane. After overnight incubation with primary antibody at 4°C, membrane was incubated with secondary antibody linked with horseradish peroxidase for 1 h at RT. Signal was detected by Immun Star HRP (Bio-Rad, Hercules, CA). The density of each band was determined using National Institutes of Health Image software and expressed as a value relative to the density of the corresponding band of the β-actin or GAPDH.

**Cell culture.** RAW264.7 cells (ATCC, Manassas, VA), a macrophage cell line, were cultured in Dulbecco’s modified Eagle’s medium (Cellgro, Manassas, VA), supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Subconfluent cells were stimulated with angiotensin II (Sigma-Aldrich) at 37°C after pretreatment of various concentrations of nicorandil for 30 min in serum-free medium. Eight hours after glibenclamide (Sigma) exposure, xanthine oxidase expression was examined. Each experiment was repeated at least four times.

**Statistical analysis.** All values are expressed as means ± SE. Statistical analysis was performed with ANOVA using Tukey’s method. A level of P < 0.05 was considered statistically significant.

**RESULTS**

Nicorandil reduces albuminuria, glomerular, and tubulointerstitial injury in the RK model of CKD. The study involved comparing the effect of either high-dose or low-dose nicorandil with no treatment in a rat RK model of CKD. Table 1 shows general characteristics of each of the three groups, as well as a sham-operated group. Four and twelve weeks after RK surgery, BP was significantly elevated in control RK rats compared with sham rats, and neither low-dose nicorandil nor high-dose nicorandil had any effects. Control RK rats also exhibited higher BUN and serum creatinine levels and lower creatinine clearances compared with sham animals, and neither dose of nicorandil improved these parameters. To further evaluate the effect of nicorandil on renal function, serum cystatin C was also measured. Consistently with serum creatinine, cystatin C was significantly higher in control RK than sham-operated rats.

**Table 1. Blood pressure, body weight, and laboratory data at 12 wk**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>RK L-NICO</th>
<th>RK H-NICO</th>
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<tr>
<td>n</td>
<td>7</td>
<td>7</td>
<td>7</td>
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<tr>
<td>Systolic blood pressure, mmHg</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0 wk</td>
<td>118 ± 3</td>
<td>116 ± 3</td>
<td>117 ± 1</td>
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<tr>
<td>4 wk</td>
<td>124 ± 3**</td>
<td>139 ± 3</td>
<td>140 ± 4</td>
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<tr>
<td>12 wk</td>
<td>130 ± 2*</td>
<td>147 ± 2</td>
<td>149 ± 2</td>
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<td>Body weight, g</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0 wk</td>
<td>230 ± 4</td>
<td>231 ± 6</td>
<td>238 ± 3</td>
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<tr>
<td>12 wk</td>
<td>541 ± 38</td>
<td>529 ± 28</td>
<td>516 ± 13</td>
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<td>Renal function at 12 wk</td>
<td></td>
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<tr>
<td>Serum urea nitrogen, mg/dl</td>
<td>12.6 ± 2.1**</td>
<td>37.0 ± 6.8</td>
<td>26.3 ± 1.5</td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.32 ± 0.03***</td>
<td>0.71 ± 0.08</td>
<td>0.62 ± 0.04</td>
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<tr>
<td>Serum cystatin C, μg/ml</td>
<td>3.56 ± 0.17***</td>
<td>5.54 ± 0.50</td>
<td>4.97 ± 0.26</td>
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<td>Creatinine clearance, ml·min⁻¹·100 g⁻¹</td>
<td>0.73 ± 0.04***</td>
<td>0.37 ± 0.04</td>
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<tr>
<td>Urine volume, ml/day</td>
<td>26.7 ± 0.9</td>
<td>35.6 ± 6.3</td>
<td>20.4 ± 2.6</td>
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<td>Urine creatinine, mg/day</td>
<td>17.9 ± 2.0</td>
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<td>18.4 ± 0.9</td>
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<td>Other data at 12 wk</td>
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<tr>
<td>Serum uric acid, mg/dl</td>
<td>3.2 ± 0.4</td>
<td>3.1 ± 0.2</td>
<td>3.2 ± 0.4</td>
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<tr>
<td>Urinary nitrate + nitrite, μmol·day⁻¹·100 g⁻¹</td>
<td>0.15 ± 0.11</td>
<td>0.27 ± 0.13</td>
<td>NE</td>
</tr>
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Values are means SE; n, no. of rats. RK, remnant kidney; L-NICO, low-dose nicorandil; H-NICO, high-dose nicorandil; NE, not examined. **P < 0.001, ***P < 0.01, *P < 0.05 vs. RK.
Nicorandil did not improve cystatin C levels compared with RK rats. In contrast, nicorandil reduced urine albumin excretion in a dose-dependent manner. In particular, the effect of high-dose nicorandil was statistically significant (Fig. 1). In addition, mild body weight loss was observed with high-dose nicorandil treatment. This finding is consistent with our laboratory’s previous studies (9, 19).

Control RK rats developed glomerulosclerosis, which was associated with an increase in collagen IV deposition and CD68(+) mononuclear cell infiltration (Fig. 2). Nicorandil dose-dependently reduced such renal injury and inflammation. Nicorandil also had a protective effect on podocytes. Control RK rats had a loss of podocytes, revealed by a lower number of WT-1-positive cells and a reduction in podocin immunoreactivity, both of which were largely prevented in RK rats administered high dose of nicorandil.

Control RK rats also developed tubulointerstitial injury with an increase in interstitial collagen III deposition and infiltration of CD68(+) and CD11b/c(+) mononuclear cells (Fig. 3). Nicorandil also reduced these effects in this model. Similar to the effects on the glomerulus, the protective effect of this compound was greater with the higher dose in tubulointerstitial injury.

Nicorandil may act by blocking oxidative stress. To investigate the mechanisms, we first looked for evidence of oxidative stress. As shown in Fig. 4, the nitrotyrosine level in renal cortex was significantly higher in RK, whereas such elevation was significantly blocked by nicorandil. Likewise, urinary 8-OHdG, another marker of oxidative stress, was elevated in cortex was significantly higher in RK, whereas such elevation of this enzyme was also prevented by nicorandil treatment. (Fig. 6, A and B). Xanthine oxidase activity was also higher in the kidneys of RK rats and was blocked by nicorandil (Fig. 6C). Likewise, intrarenal uric acid, which is a metabolite of xanthine oxidase, showed the same pattern as that observed for xanthine oxidase activity (Fig. 6D).

To identify the cells that express MnSOD, immunohistochemistry was further performed. MnSOD was predominantly expressed in podocyte as serial sections of kidney tissue indicated that MnSOD staining overlapped with WT-1 (Fig. 5F) as well as podocin (Fig. 5G). Podocytes also expressed SUR2, a subunit of KATP channel and a binding site of nicorandil (Fig. 5, H and I). As shown in Fig. 5J, the cultured human podocytes also express SUR2 and have two isoforms of SUR2, which is consistent with previous studies (2, 16, 18). These data suggest that nicorandil might directly bind to SUR2 in the podocyte to prevent MnSOD level from a reduction.

Another source of oxidative stress is the xanthine oxidase. Xanthine oxidase protein expression was significantly higher in the renal cortex of control RK rats compared with sham animals, and such elevation of this enzyme was also prevented by nicorandil treatment. (Fig. 6, A and B). Xanthine oxidase activity was also higher in the kidneys of RK rats and was blocked by nicorandil (Fig. 6C). Likewise, intrarenal uric acid, which is a metabolite of xanthine oxidase, showed the same pattern as that observed for xanthine oxidase activity (Fig. 6D). By immunohistochemistry, xanthine oxidase protein expression was markedly induced in the RK, especially in the glomerulus and interstitium. The number of positive cells was reduced with nicorandil treatment (Fig. 6, E–J). To identify the cell type expressing xanthine oxidase, we examined the expression of CD68 and CD11b/c, both of which are markers for subtypes of mononuclear cells, particularly for macrophage. We found that xanthine oxidase-positive cells were positive for both CD68 and CD11b/c (Fig. 6, K and L). Those cells also expressed SUR2 (Fig. 6, M and N). These studies suggest that nicorandil, by stimulating SUR2, blocks oxidative stress due to reducing macrophages expressing xanthine oxidase.

SUR2 mediates xanthine oxidase expression in RAW264.7 cells. We also studied the cultured macrophages to determine if SUR2 regulates xanthine oxidase expression. First, we documented that RAW264.7 cells express SUR2 (Fig. 7A). Next, we investigated if xanthine oxidase is induced by angiotensin II in macrophage, since angiotensin II is a critical mediator in RK and also is capable of upregulating xanthine oxidase in other cell types (7). As shown in Fig. 7B, angiotensin II (10^-8, 10^-6 M) induced xanthine oxidase protein expression at 4 and 8 h. In particular, the upregulation of this protein by angiotensin II at 10^-5 M reached statistical significant at 8 h. In turn, nicorandil significantly prevented the upregulation of this enzyme (Fig. 7C). Finally, glibenclamide, an inhibitor of SUR, could increase xanthine oxidase expression (Fig. 7D), suggesting SUR2 regulates xanthine oxidase expression in macrophage.

Nitric oxide released from nicorandil is not associated with its beneficial effects. Finally, we examined urine NOx (NO_3^- + NO_2^-) level as nicorandil is also a NO donor (Table 1). Nicorandil significantly increased urine NOx compared with sham or control RK rats. However, RK also has slightly higher level of urine NOx compared with sham, and thereby urine NOx was not associated with renal injury.
DISCUSSION

The novel findings in this study are that nicorandil prevented albuminuria, glomerular, and tubulointerstitial injury in a model of CKD. This beneficial effect occurred in the absence of lowering BP. The mechanism for nicorandil protection could be by reducing oxidative stress, both by increasing antioxidant systems (such as MnSOD in the podocyte) and by blocking prooxidative systems in macrophages (such as by blocking xanthine oxidase) (Fig. 8).

One of the important findings was that nicorandil could block albuminuria. This finding is consistent with the prior experimental studies (17, 19), as well as a recent report that nicorandil could reduce proteinuria in hypertensive subjects (8). Our laboratory has recently reported that nicorandil could reduce proteinuria in hypertensive subjects (8). In that study, our laboratory showed that nicorandil protects podocytes from high-glucose-induced oxidative stress by activating KATP channels.

In the present study, we used a different animal model of CKD to explore further mechanism for nicorandil protection. Consistent with our previous study, the present study demonstr-
Stratified that nicorandil protects podocytes as it prevented podocyte loss, as noted by staining for WT-1 and podocin. Importantly, we also found that nicorandil reduces oxidative stress in the podocytes by increasing its antioxidant armamentarium by increasing MnSOD levels. Given the fact that podocytes play a key role in the development of proteinuria, targeting podocytes might be a mechanism for anti-albuminuric effect of nicorandil. We also believe that nicorandil is providing such protection by activating the KATP channel, as the binding site for nicorandil is SUR2, which is expressed in the podocyte.

Another mechanism for nicorandil protection likely involves blocking tubulointerstitial injury. Again, the mechanism likely involves a reduction in oxidative stress by blocking the macrophage xanthine oxidase activity. The likelihood that blocking this enzyme may be via SUR2 was supported by our in vitro studies showing that nicorandil could block the upregulation of xanthine oxidase in cultured macrophages, and that blocking SUR resulted in a spontaneous upregulation of xanthine oxidase in this cell line.

Nicorandil failed to improve renal function in this model, despite its potent anti-proteinuric effect. One potential reason might depend on a nature of RK model. The RK model we used here is a model in which 5/6 of the kidney is surgically removed, resulting in that only 1/6 of the kidney is remnant. Hence, one might not expect the improvement of renal function in this RK model. In contrast, one might rather assume that prolonged treatment might be able to slow the progression of renal disease, even in this model (5). Hence, it remains possible that nicorandil could show renoprotection if treatment had been extended for a year.

We found that uric acid level was elevated in the kidney, but not in the serum. Importantly, uric acid, a metabolite of xanthine oxidase, is also a prooxidant and may play a role in renal disease (11–14). Hence, an elevation of intrarenal uric acid, observed in this study, could provide another source of oxidative stress. Since nicorandil blocks xanthine oxidase activity, we were not able to determine whether the reduction in oxidative stress was due to a reduction in oxidants produced by xanthine oxidase or by reducing uric acid itself.

Fig. 4. Nicorandil reduces oxidative stress in the RK. A: Western blotting with renal cortex demonstrates that nitrotyrosine level are significantly higher in RK compared with sham, whereby such increase in nitrotyrosine is significantly attenuated by H-NICO. B: quantification of nitrotyrosine. C: likewise, urine 8-hydroxy-2’-deoxyguanosine (8-OHdG), another marker of oxidative stress, is also higher in RK compared with sham and H-NICO. D: the expression of heme oxygenase-1 is significantly higher in RK, while such elevation is blocked by nicorandil treatment. Values are means ± SE. **P < 0.01 vs. RK, *P < 0.05 vs. RK.
Fig. 5. Nicorandil protects podocyte manganese SOD (MnSOD) in the RK. Western blotting with renal cortex shows that MnSOD protein expression is reduced in RK compared with sham. A: however, such reduction of MnSOD is attenuated by H-NICO treatment. B: quantification of this Western blotting. Immunohistochemistry demonstrated that MnSOD (arrow) is expressed in glomerulus. The number of positive cells is fewer in RK (D) compared with sham (C) and H-NICO (E). F, G, H, and I: immunohistochemistry with serial sections. In F and G, MnSOD (brown color indicated by arrow) is overlapped with WT-1 and podocin (blue indicated by arrow), respectively. In H and I, sulfonylurea receptor 2 (SUR2) (brown indicated by arrow) is overlapped with WT-1 and podocin (blue indicated by arrow), respectively. J: Western blotting with cultured human podocytes shows that podocytes express two isoforms of SUR2. Values are means ± SE. ***P < 0.001 vs. RK, *P < 0.05 vs. RK. Scale bar indicates 50 μm.
Fig. 6. Nicorandil blocks xanthine oxidase (XO) in the infiltrating mononuclear cells of the RK. Western blotting with renal cortex shows that XO protein expression is higher in RK compared with sham. A: however, the upregulation of XO is attenuated by H-NICO treatment. B: quantification of this Western blotting. C: XO activity is also higher in RK compared with sham, while H-NICO significantly inhibits such activation. D: likewise, intrarenal uric acid level is higher in RK than that in sham and nicorandil treatment. Consistent with Western blotting, immunohistochemistry demonstrated that XO (arrow) is induced in glomerulus (F) and interstitium (I) in the RK compared with sham (E and H), while nicorandil blocks these changes (G and J). K, L, M, and N: immunohistochemistry with serial sections. In K and L, XO (brown color indicated by arrow) is overlapped with CD68 and CD11b/c (blue indicated by arrow), respectively. In M and N, infiltrating cells are positive for SUR2 (brown indicated by arrow), most of which are also positive for CD68 (M) and CD11b/c (N). Values are means ± SE. **P < 0.01 vs. RK, *P < 0.05 vs. RK. Scale bar indicates 50 μm.

Fig. 7. SUR2 mediates XO expression in RAW264.7 cells. A: Western blotting of SUR2 in the cultured human podocytes, showing the SUR2 protein expression. B: Western blotting shows that angiotensin II (AII; 10^{-8} M–10^{-6} M) induces XO protein expression in RAW264.7 cells. Quantification using National Institutes of Health image demonstrated that XO protein expression is dose-dependently induced by AII at 8 h. C: at 8 h, the upregulation of XO in response to 10^{-7} M AII is significantly blocked by nicorandil at 10^{-6}–10^{-4} M. D: blocking SUR2 by glibenclamide at 10^{-7}–10^{-5} M significantly induces XO in RAW264.7 cells at 8 h. CON, control. Values are means ± SE. ***P < 0.001, **P < 0.01, *P < 0.05.
In conclusion, nicorandil is a podocyte protectant that preserves podocyte number and reduces urine albumin excretion in a rat model of CKD. The protective mechanism involves a reduction in oxidative stress, likely stimulated via $K_{ATP}$ channel.

GRANTS
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DISCLOSURES
T. Nakagawa and R. J. Johnson are listed as inventor on a patent application proposing nicorandil as a treatment for acute and chronic kidney disease, which has been licensed by Cardero. T. Nakagawa, R. J. Johnson, and G. F. Schreiner are stock owners for Cardero Therapeutics.

AUTHOR CONTRIBUTIONS
Y.T., K.T., W.K., and T.N. performed experiments; Y.T. and T.N. analyzed data; Y.T. and T.N. interpreted results of experiments; Y.T. prepared figures; Y.T., K.T., W.K., S.U., G.F.S., R.J.J., and T.N. approved final version of manuscript; S.U., R.J.J., and T.N. edited and revised manuscript; G.F.S., R.J.J., and T.N. conceived and design of research; T.N. drafted manuscript.

REFERENCES


