Nicorandil as a novel therapy for advanced diabetic nephropathy in the eNOS-deficient mouse

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Nicolandil is the sodium salt of nicorandil, an orally available drug. This is a clinical proven agent that causes vasodilation by dual action: one is releasing nitric oxide (NO) and the other is a selective ATP-dependent K channel activator. We hypothesized that nicorandil may have a beneficial role in treating diabetic nephropathy. We administered nicorandil to a model of advanced diabetic nephropathy (the streptozotocin-induced diabetes in mice lacking endothelial nitric oxide synthase, eNOSKO) in order to test the hypothesis. We found that nicorandil did not affect blood glucose levels, blood pressure, or systemic endothelial function, but significantly reduced proteinuria and glomerular injury in diabetic eNOS KO mice treated with either nicorandil or vehicle. Mice were treated for 8 wk. Histology, blood pressure, and renal function were determined. Additional studies involved examining the effects of nicorandil on cultured human podocytes. Here, we found that nicorandil did not affect blood glucose levels, blood pressure, or systemic endothelial function, but significantly reduced proteinuria and glomerular injury (mesangiosis and glomerulosclerosis). Nicorandil protected against podocyte loss and podocyte oxidative stress. Studies in cultured podocytes showed that nicorandil likely protects against glucose-mediated oxidant stress via the ATP-dependent K channel as opposed to its NO-stimulating effects. In conclusion, nicorandil may be beneficial in diabetic nephropathy by preserving podocyte function. We recommend clinical trials to determine whether nicorandil may benefit diabetic nephropathy or other conditions associated with podocyte dysfunction.

METHODS

Experimental Protocols

All animal experiments were performed in accordance with the Animal Care and Use Committee of the University of Colorado. Male C57BL/6J-Nos3tm1nc mice (eNOSKO mice) were purchased from Jackson Laboratory (Bar Harbor, ME) at 8 wk of age. Mice were fed a standard laboratory chow ad libitum. Diabetic nephropathy was induced by intraperitoneal injections of streptozotocin (50 mg·kg−1·day−1 for 5 consecutive days) dissolved in 10 mM citrate buffer, pH 4.5 (5). Diabetes was defined as nonfasting blood glucose >250 mg·dl−1 using a blood glucose meter (One Touch Ultra; Life Scan, Milpitas, CA). While no mice developed diabetes at day 7 after streptozotocin administration, 33, 72, and 85% of mice became diabetic at 2, 3, and 4 wk, respectively. These mice were chronically treated with nicorandil and were compared with diabetic wild-type mice (12). Thus another therapeutic option is needed in diabetes with endothelial dysfunction.

Organic nitrates are used to provide nitric oxide (NO), which is expected to improve endothelial function and cardiovascular disease. However, long-term effects are limited due to the development of tolerance (35). In addition, organic nitrates are found to rather exacerbate endothelial dysfunction due to inducing oxidative stress (35). Hence organic nitrates as previously tested are unlikely to provide a benefit in diabetic nephropathy.

Nicorandil, or 2-[(pyridin-3-ylcarbonyl)amino]ethyl nitrate, is a clinically proven antianginal agent that causes vasodilation by dual action: one is releasing NO and the other is a selective ATP-dependent K channel activator. We hypothesized that nicorandil may have a beneficial role in treating diabetic nephropathy. In this study, we tested the effect of nicorandil in the diabetic mice in which eNOS production is permanently disturbed. Data demonstrate that nicorandil does not result in an improvement in systemic endothelial dysfunction in contrast to expectation. However, we found that nicorandil directly reduced oxidative stress in podocytes via the ATP-dependent K channel.

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respective. Only mice which developed hyperglycemia at 4 wk were included in the study. Mice were divided into four subgroups: 1) a nondiabetic group, 2) a nicorandil-treated nondiabetic group, 3) a diabetic group, and 4) a nicorandil-treated diabetic group (n = 8/group). At 4 wk when the onset of diabetes was confirmed in all animals, 30 mg/kg of nicorandil (Chugai Pharmaceutical, Tokyo, Japan) was started. To constantly administer the same amount of nicorandil, the concentration of nicorandil in the drinking water was adjusted every 4 days along with as per the water intake volume. Water bottles were monitored daily throughout the study to ensure no leakage occurred. Systolic blood pressure was measured every other week using a tail-cuff sphygmomanometer (Visitek BP-2000; Visitek Systems, Apex, NC). Urine was collected overnight using metabolic cages (Techniplast, Exton, PA). All the mice were euthanized 8 wk after starting nicorandil treatment to obtain blood samples and kidney tissues.

**Laboratory Studies**

Urine albumin, urine 8-hydroxy-2-deoxyguanosine (8-OHdG), and urine creatinine were measured with Albuwell M (Exocell, Philadelphia, PA), an OxiSelect Oxidative DNA Damage ELISA Kit (Cell Biolabs, San Diego, CA), and Creatinine LiquiColor Test (Enzymatic Methodology; Stanbio, Boerne, TX), respectively. Serum creatinine concentration was analyzed with HPLC-tandem mass spectrometry (MS/MS; Applied Biosystems 3200 Qtrap). Creatinine and [2H3]creatinine (CDN isotopes) were detected in the multiple reaction monitor of nicorandil. Only mice which developed hyperglycemia at 4 wk were included in the study. To constantly administer the same amount of nicorandil, the concentration of nicorandil in the drinking water was adjusted every 4 days along with as per the water intake volume. Water bottles were monitored daily throughout the study to ensure no leakage occurred. Systolic blood pressure was measured every other week using a tail-cuff sphygmomanometer (Visitek BP-2000; Visitek Systems, Apex, NC). Urine was collected overnight using metabolic cages (Techniplast, Exton, PA). All the mice were euthanized 8 wk after starting nicorandil treatment to obtain blood samples and kidney tissues.

**Table 1. General characteristics of nondiabetic and diabetic mice**

<table>
<thead>
<tr>
<th></th>
<th>NonDM</th>
<th>NonDM + Nicorandil</th>
<th>DM</th>
<th>DM + Nicorandil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>26.9 ± 2.6</td>
<td>27.3 ± 1.4</td>
<td>22.6 ± 1.9*</td>
<td>22.5 ± 1.7*</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>119.5 ± 7.0</td>
<td>118.1 ± 10.2</td>
<td>376.3 ± 85.4*</td>
<td>353.0 ± 24.9*</td>
</tr>
<tr>
<td>Kidney wt/body wt ratio (×10⁻⁶)</td>
<td>4.5 ± 0.4</td>
<td>4.4 ± 0.7</td>
<td>7.4 ± 1.7*</td>
<td>7.3 ± 0.7*</td>
</tr>
<tr>
<td>Urine albumin/creatinine ratio (×10⁻¹)</td>
<td>10.6 ± 6.7</td>
<td>9.2 ± 4.6</td>
<td>57.2 ± 12.4*</td>
<td>17.2 ± 10.4*†</td>
</tr>
<tr>
<td>Ccr, ml/min</td>
<td>0.22 ± 0.19</td>
<td>0.21 ± 0.14</td>
<td>0.34 ± 0.10</td>
<td>0.39 ± 0.27</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 8/group. NonDM, nondiabetic; DM, diabetic; Ccr, creatinine clearance. *P < 0.01 vs. NonDM. †P < 0.05 vs. DM.
Fig. 1. Glomerular disease in diabetic mice lacking endothelial nitric oxide synthase (eNOSKO). Representative light microscopic appearance of glomerular lesions is shown (periodic acid-Schiff staining, ×400 original magnification). Compared with nondiabetic (A), diabetic eNOSKO mice show mesangial expansion (B), mesangiolysis (C), and glomerulosclerosis (D). Nicorandil treatment significantly reduces the incidences of mesangiolysis (L) and glomerulosclerosis (M) in diabetic eNOSKO mice. Immunohistochemistry for mesangial accumulation of type IV collagen for nondiabetes (E), nondiabetes with nicorandil (F), diabetes (G), and diabetes with nicorandil (H) in eNOSKO mice (×400 original magnification) is shown. Type IV collagen-positive area in the glomerulus is significantly reduced by nicorandil treatment in diabetic eNOSKO mice (N). Induction of diabetes also results in an increase in the number of F4/80-positive cells in the glomerulus (J, arrow) compared with nondiabetic mice (I). Nicorandil exhibits an inhibitory effect on the glomerular infiltration of monocytes/macrophages in diabetic eNOSKO mice (O). Values are means ± SD; n = 8/group. *P < 0.01 vs. nondiabetes. †P < 0.05 vs. diabetes.
The injections of streptozotocin resulted in marked hyperglycemia and an increased kidney size (a ratio of kidney/body weight) accompanied by a significant loss of body weight in the eNOSKO mice. Urinary albumin excretion and creatinine clearance were also increased, consistent with early hyperfiltration. Nicorandil significantly reduced albuminuria by 70% in diabetic eNOSKO mice while no effects were observed on renal function (Table 1).

Glomerular Histology

Compatible with previous reports (10, 22), diabetic conditions caused glomerular hypertrophy and mesangial expansion accompanied by more severe glomerular lesions such as mesangiolysis or glomerulosclerosis in eNOSKO mice compared with nondiabetic glomeruli (Fig. 1, A–D). Nicorandil treatment significantly reduced the development of mesangiolysis and glomerulosclerosis (Fig. 1, L and M, respectively). Similarly, type IV collagen deposition, a marker of mesangial matrix expansion, was markedly increased in diabetic eNOSKO mice (Fig. 1G) compared with nondiabetic groups (Fig. 1, E and F) while it was reduced by nicorandil treatment (Fig. 1, H and N). Similarly, diabetic conditions induced F4/80-positive cell infiltration in the glomeruli of diabetic eNOSKO mice whereas nicorandil significantly reduced the number of infiltrating cells (Fig. 1, I, J, and O).
Investigations of Mechanisms Whereby Nicorandil is Protective

Effect on blood pressure. Blood pressure tended to be higher in diabetic eNOSKO mice after 2 wk compared with nondiabetic mice, but the difference did not reach statistical significance (Fig. 2A). Nicorandil did not significantly lower blood pressure in either diabetic or nondiabetic groups during the 8-wk treatment period.

Effects on endothelial function. To evaluate whether nicorandil improves endothelial function, serum markers for endothelial dysfunction, P-selectin and ICAM-1, were measured. Serum P-selectin and serum ICAM-1 levels were significantly elevated in diabetic eNOSKO mice; however, nicorandil did not improve either marker (Fig. 2, B and C). These data suggest that nicorandil likely failed to improve systemic endothelial function in eNOSKO mice.

Fig. 3. Expression of podocin and WT-1 in diabetic eNOSKO mice. The expression of podocin, a podocyte-specific marker, in glomeruli was determined by immunohistochemistry for nondiabetes (A), nondiabetes with nicorandil (B), diabetes (C), and diabetes with nicorandil (D) in eNOSKO mice (×400 original magnification). The reduction of the podocin-positive area in glomeruli as detected by image analysis is significantly inhibited by nicorandil treatment in diabetic eNOSKO mice (G). Immunohistochemistry for WT-1, another marker of podocytes, is also shown in nondiabetic mice (E) and diabetic mice (F). Diabetes induces a decrease in the number of WT-1-positive podocytes in glomeruli (F) compared with nondiabetic mice (E). The number of WT-1-positive podocytes in glomeruli, which is determined by the Weibel-Gomez method, is shown (H). Nicorandil treatment significantly prevents the decrease in podocyte number in diabetic eNOSKO mice (H). Albuminuria is negatively correlated with WT-1-positive podocytes (I). Values are means ± SD; n = 8/group. *P < 0.01 vs. nondiabetes. †P < 0.05 vs. diabetes.
Effects on podocytes. One of the key findings in diabetic nephropathy is a loss of podocytes (27), which has been hypothesized to increase the risk for both proteinuria as well as glomerulosclerosis (13). Thus the marked benefit of nicorandil on proteinuria in the absence of blood pressure control raised the hypothesis that nicorandil might be having a specific effect on the podocytes. To assess the effect on podocytes, we performed immunohistochemistry with the tissues for WT-1 (which marks podocyte nuclei) and podocin (a podocyte specific marker). It was shown that diabetes markedly decreased the expression of podocin (Fig. 3C) compared with nondiabetic groups (Fig. 3, A and B). However, nicorandil partially restored its expression (Fig. 3, D and G). Similarly, the number of WT-1-positive podocytes per glomelurus was decreased in diabetic eNOSKO mice whereas nicorandil also significantly prevented the decrease in the number of podocytes (Fig. 3, E, F, and H). As shown in Fig. 3I, urinary albumin excretion in diabetic animals with/without nicorandil was negatively correlated with podocyte number in this study.

The loss of podocytes was associated with increased oxidative stress in podocytes. Indeed, 8-OHdG (Fig. 4A) and nitrotyrosine (Fig. 4C) were increased in podocytes of diabetic mice, and it appeared to be inhibited in nicorandil-treated mice (Fig. 4, B and D). Consistent with these data, urinary levels of 8-OHdG (Fig. 4E) as well as renal cortical levels of nitrotyrosine (Fig. 4, F and G) were also elevated in diabetic eNOSKO mice and were reversed by nicorandil treatment (Fig. 4, F and G). Perhaps the suppression of oxidative stress by nicorandil could account for the reduction in nitrotyrosine.

Effects of nicorandil on podocytes in vivo. The remarkable protective effect of nicorandil on podocytes in diabetic eNOSKO mice led to the hypothesis that nicorandil might have direct effects on the podocyte. Since nicorandil is capable of donating NO, we initially assumed that its protective effects on podocytes could be due to biological actions of NO. Hence, we examined the distribution of cGMP, as a second messenger in NO signaling. However, in both nondiabetic (Fig. 5A, left) and diabetic eNOSKO mice (Fig. 5A, right), cGMP was not predominantly detected in podocyte, but it was positive in tubular epithelial cells.

Given these facts, we then hypothesized that the effect of nicorandil on podocytes might be via effects on SUR-2 of the ATP-dependent K channel (28, 31). As shown in Fig. 5B, SUR-2 expression is likely expressed by both mesangial cells and podocytes in vivo in the normal mouse, which was not altered in diabetic eNOSKO mice. Double staining of SUR-2 with synaptopodin (a marker of podocytes) confirmed the expression of the ATP-dependent K channel in podocytes. In contrast, SUR-2 expression was barely detected in tubules. Thus it is likely that nicorandil directly interacts with podocytes via the ATP-dependent K channel rather than NO-cGMP signaling.

By using human cultured podocytes, we verified the mRNA expression of SUR-2 in differentiated immortalized podocytes. RT-PCR detected the expression of both subtypes, SUR-2A and SUR-2B in the podocytes (Fig. 6A).

Reduced production of ROS by nicorandil in cultured podocytes. Finally, we examined the direct effect of nicorandil on the intracellular production of ROS in cultured podocytes. Using the DCF detection assay, we assessed the amount of ROS as the conversion from H2DCFDA to DCF. The intensity of DCF fluorescence was markedly increased in podocytes
with HG (Fig. 6D) compared with NG or NG+M (Fig. 6, B and C). However, nicorandil treatment significantly reduced the DCF intensity in HG (Fig. 6, E and F). Given that excess production of ROS led to podocyte loss in vivo, we finally examined the cultured podocyte number in NG or HG condition using an MTT assay. A reduction in podocyte number, which was defined as a decrease in the formation of reduced MTT, was observed in HG whereas nicorandil significantly preserved its reaction (Fig. 6G). These results indicate that podocyte protection by nicorandil observed in the diabetic mice may be attributed to a reduction in the intracellular production of ROS.

**DISCUSSION**

In the present study, we examined the effects of nicorandil in diabetic eNOSKO mice in which endothelial NO production is genetically and therefore permanently blocked. Here, we assumed that NO released from nicorandil might compensate for a deficiency of endothelial NO and ameliorate the progression of advanced diabetic nephropathy. While nicorandil exhibited a protective effect on proteinuria and glomerular histology, such protection was unlikely due to NO donation or improvement of endothelial function but rather to podocyte protection. In particular, podocytes are found to express an ATP-dependent K channel, which therefore is able to be stimulated by nicorandil. Our in vitro study suggested that nicorandil could reduce oxidative stress through its binding to the ATP-dependent K channel.

Podocytes play a pivotal role in maintaining the integrity of the glomerular filtration barrier, and therefore podocyte injury is thought to lead to the development of albuminuria. While podocyte damages can be caused by diabetes, it might be due to the ability of glucose to increase oxidative stress (33, 34). We also previously reported that a lack of endothelial NO results in podocyte injury in the mouse (24), and therefore endothelial dysfunction could contribute to the impairment of podocyte function in diabetes. Given these facts, targeting oxidative stress in the podocytes could be a therapeutic option to block diabetic nephropathy.

Nicorandil, a nicotinamide nitrate, exerts vasodilatory effects and therefore has been used clinically for the treatment of ischemic heart diseases (3). Such vasodilatory effects are due to the ability of nicorandil to donate NO and consequently to activate the soluble guanylate cyclase (sGS)-cGMP pathway. This compound is also known to stimulate the ATP-dependent K channel, predominantly exists in the mitochondria, to increase transmembrane potassium conductance and induce vasodilatation. In addition, an opening of the ATP-dependent K channel also likely contributes to cardiac protection owing to the development of ischemic preconditioning, a phenomenon whereby intermittent bouts of transient ischemia render the heart more resistant to future ischemic insults (20). Recently, it has been reported that nicorandil exhibits some protections in kidney disease, including anti-Thy.1 antibody-induced mesangial proliferating glomerulonephritis (32) and ischemic-reperfusion injury in the rat (30). While the mechanism of renopro-
Protective efficacy of nicorandil has not yet been elucidated, mechanisms likely involve the protection of mitochondrial function and prevention of cell apoptosis (8).

SUR-2, a composing subunit of the ATP-dependent K channel, is found to be expressed in many tissues, including pancreatic islet cells, heart, skeletal muscle, vascular smooth muscle, and brain (29). The current study is, to our knowledge, the first documentation showing that SUR-2 is expressed in podocytes. While nicorandil has a dual function in which it is able to donate NO and stimulate the ATP-dependent K channel, the protective effect on podocytes is likely through opening of ATP-dependent K channels (37) because a cGMP signal, as a second messenger of NO, was not detected in the glomerulus. With respect to the localization of SUR-2 in the kidney, Zhou et al. (38) found that SUR-2 is expressed in tubular epithelial cells, yet their finding is distinct from our results. However, such discrepancy could be explained by the difference in species or antibody used.

Over the past decade, both inorganic and organic nitrates, as NO donors, have been tested for cardioprotection in subjects with coronary artery disease. However, unexpected outcomes have been documented in many clinical trials. In fact, many of these compounds paradoxically produce oxidative stress, induce endothelial dysfunction, and result in nitrate tolerance (17–19). Precise mechanisms for nitrate tolerance are being uncovered (16), but it is likely that oxidative stress derived from other nitrate compounds impairs aldehyde dehydrogenase to cause nitrate tolerance in mitochondria (15, 21). In contrast, clinical studies, albeit a limited number of investigations, documented (1) that unlike other nitrates, nicorandil does not seem to induce tolerance in its ability to donate NO (26). A precise mechanism for this unique and favorable effect of nicorandil remains to be determined. The antioxidative function of nicorandil could account for this benefit.

A major finding in this study is our demonstration that advanced diabetic nephropathy in which endothelial dysfunction is not reversible could be prevented by nicorandil treatment in mice. Although it did not seem to improve systemic endothelial function, chronic administration of nicorandil reduced oxidative stress by stimulating the ATP-dependent K channel and consequently protected podocytes independently of donating NO. However, we also have to mention that this benefit of nicorandil was mild, and therefore further studies are required to determine how to completely block the progression of advanced diabetic nephropathy.

The authors note several limitations in this study, including a lack of groups with wild-type mice. Perhaps it might be better to evaluate the effect of nicorandil in diabetic wild-type mice as such a benefit might be expected. Another point is that glomerular endothelial function has not yet been elucidated in this model. While nicorandil failed to reduce serum P-selectin and ICAM1 levels, we documented the favorable effect on

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**Fig. 6. Expression of SUR-2 and production of oxidative stress in the cultured podocytes.**

RT-PCR shows the mRNA expression of both subtypes of SUR-2, SUR-2A and SUR-2B, in differentiated immortalized podocytes (A). A DCF signal is measured as reactive oxygen species (ROS) in differentiated immortalized podocytes (B–E). Podocytes were incubated with with normal glusose (NG), NG+mannitol (NG+Man), high glucose (HG), or HG+nicorandil (HG+Nico) for 72 h. Compared with NG (B) or NG+Man (C), immunofluorescent intensity for DCF is markedly higher in HG (D). Nicorandil treatment significantly inhibited the ROS signal in response to HG (E). Quantification of DCF signals is shown in F. An MTT assay indicates that HG reduces podocyte number whereas nicorandil prevents its reaction (G). Values are means ± SD; n = 4 for DCF assay and n = 6 for MTT assay in each group. *P < 0.01 vs. nondiabetes. †P < 0.05 vs. diabetes.
mesangiolysis, which is believed to be caused by glomerular endothelial dysfunction. Hence it might be conceivable that nicorandil could protect glomerular endothelial cells from diabetic insults. Further study is needed to clarify these issues, in particular by use of diabetic wild-type mice.

In conclusion, we demonstrate that nicorandil ameliorated glomerular disease in streptozotocin-induced diabetic eNOSKO mice. The therapeutic efficacy of nicorandil observed in the present study suggests the potential of this drug as an additional option in treating diabetic nephropathy in patients with endothelial dysfunction.

**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


