Drug Discovery for Overcoming Chronic Kidney Disease (CKD): Development of Drugs on Endothelial Cell Protection for Overcoming CKD

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Abstract. Chronic kidney disease (CKD) is becoming a major public health problem worldwide. It is important to protect endothelial function in CKD treatment because injury of the endothelium is a critical event for the generation and progression of CKD. Recently, clinical studies showed that nifedipine, an antihypertensive drug, acts as a protective agent of endothelial cells (ECs). Nifedipine is reported to partially decompose to a nitrosonifedipine that has high reactivity against lipid-derived radicals in vitro. However, it is still unclear whether nitrosonifedipine is a biologically active agent against endothelial injury. We observed that nitrosonifedipine was converted to radical form by reaction with cultured ECs. The cumene hydroperoxide mediated cytotoxicity was reduced by nitrosonifedipine in cultured human glomerular ECs (HGECs). Also nitrosonifedipine suppressed the expression of TNF-α–induced intercellular cell adhesion molecule-1 in HGECs. Chronic administration of Nω-nitro-L-arginine methyl ester (L-NAME) caused systemic arterial hypertension, endothelial injury, and renal dysfunction. In L-NAME–induced hypertensive rats, nitrosonifedipine treatment improved not only the acetylcholine-induced vasodilation of the aortic rings, but also renal dysfunction such as increasing the levels of serum creatinine and urinary protein excretion. Our preliminary data suggest that nitrosonifedipine is a new and useful drug for the treatment of CKD involving ameliorating effects on EC disorder.

Keywords: chronic kidney disease (CKD), endothelial dysfunction, nitrosonifedipine, antioxidative effect

Introduction

Chronic kidney disease (CKD) has been increasingly recognized as a major public health problem in Japan and worldwide. The endothelium is a fundamental layer in the arterial wall for the local regulation of flow to organs like the kidney, and injury of it can be a critical insult for the generation and progression of CKD (1, 2). Therefore it is necessary to protect endothelial function in CKD treatment. Nifedipine, an L-type Ca2+-channel blocker, has been generally used in treatment for hypertension or angina pectoris. Clinical studies have shown that nifedipine has protective effects on organs of circulation such as prevention of renal dysfunction, cardiac events, and coronary atherosclerosis (3–6). Nifedipine is known to increase endothelial nitric oxide (NO) bioavailability mediated by its antioxidative effects and up-regulation of endothelial NO synthase (NOS) expression and activity (7, 8). In addition, nifedipine exerts a direct effect on endothelial cell (EC) permeability by inhibiting protein kinase C and attenuates NF-κB activation, which are each independent of its calcium channel–blocking activity (9, 10). These observations suggest that nifedipine has organ protective
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effects including renoprotection, at least in part, beyond its antihypertensive actions. However its detailed mechanisms have not been identified as yet.

Nifedipine is extremely light-sensitive and can be converted to its nitroso analog, nitronifedipine, under normal room light levels (Fig. 1) (11), and also this reaction can occur catalytically in the liver without illumination (12). The ability of calcium-channel blockade of nitronifedipine is quite weak compared with that of nifedipine (13). Nitronifedipine was reported to give high reactivity against lipid-derived radicals in vitro, and we and other researchers had demonstrated that nitronifedipine radical participated in radical scavenging activity (14). We hypothesized that nitronifedipine is a possible biologically active agent through its antioxidative effect. Therefore, we investigated 1) whether ECs convert nitronifedipine into nitronifedipine radical, 2) whether nitronifedipine has an EC protective activity in vitro, and 3) whether nitronifedipine shows organ protective effects including renoprotection in vivo.

Radical scavenging activity of the nitronifedipine radical

Nifedipine is reported to partially decompose to nitronifedipine by light exposure, and it possesses a considerably high antioxidative activity against lipid-derived radicals in vitro (11, 14). However, the mechanism of action for how nitronifedipine exerts antioxidative activity is still unclear. To address this question, we synthesized nitronifedipine as follows: Nifedipine solution (10 mM) in methanol was irradiated with a halogen light (500W, Kodak EKTAGRPHIC III E PLUS PROJECTOR; Eastman Kodak Co., Rochester, NY, USA) for about 18 h with constant stirring. The completion of photodegradation was monitored by high performance liquid chromatography with a UV detector (260 nm) using water:methanol (40:60) as a mobile phase. Exposed nifedipine solution was evaporated nearly to dryness and recrystallized several times from methanol. When we measured the synthesized nitronifedipine by electron paramagnetic resonance (EPR) spectroscopy, it appeared that the compound was the non-radical form (Fig.1B). However, when this compound was co-incubated with human umbilical vein endothelial cells (HUVECs), formation of nitronifedipine radical was observed in time-dependent manner, and this reached to a plateau level at 6 h incubation. Fujii et al. reported that nitronifedipine gave nitronifedipine radical in the presence of linoleic acid, suggesting that unsaturated fatty acids are integral to the nitronifedipine radical generation (12). Indeed, we found that nitronifedipine was converted into nitronifedipine radical in the presence of unsaturated fatty acids (linolenic, linolenic, arachidonic, eicosapentaenoic, and docosahexaenoic acid), whereas saturated fatty acids (palmitic and stearic acid) were not (data not shown). These findings suggest that the biomembrane layer of ECs including unsaturated fatty acids can be responsible for the generation of nitronifedipine radical from nitronifedipine. Next, to investigate whether nitronifedipine radical has radical scavenging ability, we measured the interaction between nitronifedipine and 1,1-diphenyl-2-picrylhydrazyl (DPPH) by EPR spectroscopy as described previously (15). DPPH is a stable free radical, and the disappearance of its EPR signal in the presence of test compound indicates the direct free radical scavenging activity of the test compound. When 0.1 mM DPPH was mixed with a solution containing 0.1 mM nitronifedipine and 0.1 mM unsaturated fatty acid, the DPPH radical was significantly reduced (relative DPPH signal intensity: 57 ± 5%, 55 ± 4%, and 50 ± 5% for linolenic, linolenic, and arachidonic acid, respectively) compared to the control. The reaction mixture containing nitronifedipine and saturated fatty acids gave no DPPH radical scavenging activity (3 ± 2% and 2 ± 2% for palmitic and stearic acid, respectively), suggesting that the lipid bilayer of the cell membrane facilitates the formation of nitronifedipine radical, which in turn acts as an antioxidative compound in ECs.

Effects of nitronifedipine on the cytotoxicity in ECs

ECs form the barrier between the vascular space and the surrounding tissues. They regulate the blood stream, synthesis and release of nitric oxide, and vessel permeability, which are responsible for the regulation and control of the continuous flow of plasma and blood cells. During inflammation, ECs become activated and participate in inflammatory responses through the expression of pro-inflammation genes, including cyto-
kines, chemokines and growth factors (16). Although endothelial dysfunction occurs in many different disease processes, oxidative stress can be identified as a common denominator (17, 18). Reactive oxygen species (ROS) are leading candidates in the etiology of endothelial dysfunction and ensuing cardiovascular disease (19). To evaluate the protective effects of nitrosonifedipine on ECs by its radical scavenging ability, we studied the effects of nitrosonifedipine on the oxidative cytotoxicity induced by cumene hydroperoxide (CuOOH) in cultured human glomerular ECs (HGECs). CuOOH is a lipid soluble hydroperoxide which is widely used as a source of ROS (20). CuOOH treatment significantly decreased cell viability in HGECs (100 μM; 55 ± 6% of control, evaluated by MTT assay), which was significantly recovered by the addition of nitrosonifedipine (10 μM, 90 ± 9% of control), but not by nifedipine (10 μM, 61 ± 14% of control). These results suggest nitrosonifedipine improves the cytotoxicity based on membrane-disordering potency by CuOOH in ECs. It is recognized that inflammation stimulates adhesion molecule expression at the site of endothelial dysfunction. Tumor necrosis factor-α (TNF-α) is one of the major inflammatory cytokines that mediates systemic inflammation and immune responses (21). A major site of action of TNF-α for these effects is the vascular endothelium (22), where it induces inflammatory responses by enhancing expression of cell adhesion molecules, such as E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular cell adhesion molecule-1 (ICAM-1), and secretion of inflammatory mediators (23–25). These molecules mediate transmigration of leukocytes from the blood stream into vascular endothelium (26). In order to investigate whether nitrosonifedipine attenuates ICAM-1 expression induced by TNF-α in ECs, the following experiments were performed. ICAM-1 expression was determined by Western blotting analysis as described previously (27), and it was increased time-dependently (0 to 12 h) by TNF-α (10 ng/ml) in ECs. Nitrosonifedipine (10 μM) treatment significantly diminished the expression of ICAM-1 induced by TNF-α (8 ± 1% of TNF-α treatment), but nifedipine showed no effects (10 μM, 102 ± 2% of TNF-α treatment) (Fig. 2). This phenomenon was also observed in HGECs by additional diphenyleneiodonium chloride (DPI), an inhibitor for NADPH oxidase (53 ± 2% of TNF-α treatment), suggesting that that ROS are responsible for the ICAM-expression of ECs (28). Taking this evidence together with our present study, the suppression of TNF-α-induced ICAM-1 expression by nitrosonifedipine is suggested to be involved in its ROS scavenging ability in ECs. Further studies to elucidate the precise molecular mechanisms by which nitrosonifedipine improves endothelial dysfunction via inhibition of ROS generation will be needed.

**Effects of nitrosonifedipine on organ protection in L-NAME–induced hypertensive rats**

Endothelial dysfunction is caused by a disturbance in the balance between harmful ROS and beneficial NO in the vascular wall (29, 30). Endothelial dysfunction contributes to the atherosclerotic process and is closely associated with the pathological development or events in hypertension, heart failure, and kidney disease (31). Pharmacological long-term blockade of NO synthesis by the chronic administration of Nω-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS, produces systemic arterial hypertension, vascular structural change, and renal dysfunction (19, 32). In order to demonstrate a protective effect of nitrosonifedipine for L-NAME–induced hypertensive rats, we conducted the following experiments. Male SD rats were divided into three groups [control group, L-NAME group, and L-NAME + nitrosonifedipine (30 mg/kg, intraperitoneally) group]. L-NAME (1 g/l) was administered in drinking water for three weeks. Daily intake of L-NAME was estimated to be 20–30 mg per rat (33). At the starting point of the experiment, basal systolic blood pressure (SBP) was up to 105 mmHg, and there were no significant differences among groups. L-NAME treatment caused a progressive increase in SBP from the first week, reaching the value of 146 ± 7 mmHg at week 3, which was consistent with our previous findings (33). Co-administration of nitrosonifedipine tended to decrease the SBP, although no significant difference was observed in SBP between the L-NAME-treated group and L-NAME + nitrosonifedipine group because of the weak L-type Ca2+–channel–blocking activity of nitrosonifedipine (13) (Table 1). It has also been reported that endothelium-dependent relaxation was decreased in the aorta from L-NAME hypertensive rats due to endothelial dysfunction (34, 35).
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Then we determined whether nitrosonifedipine treatment attenuated the endothelial dysfunction induced by L-NAME treatment. Thoracic aortae were dissociated from rats and then placed in an organ bath for the measurement. Exposure to cumulative concentrations of acetylcholine (ACh) (from 10⁻⁹ to 10⁻⁶ M) resulted in a marked relaxation (75 ± 2%, maximal relaxant effect, expressed as percentage of phenylephrine-induced contractions of the aortic rings from the control group). The sensitivity to Ach of the aortic rings from rats given L-NAME alone was significantly reduced, corresponding to only 19 ± 7% of the control at a high concentration of ACh (10⁻⁶ M), as expected. Contrary to the result of L-NAME alone, aortic rings from rats given L-NAME + nitrosonifedipine showed a significant restoration of the vasodilation, with the maximal relaxant effect being 51 ± 9%. These data clearly demonstrated that nitrosonifedipine has beneficial effects on the endothelial vasodilator function in a model of chronic NO inhibition. ICAM-1 expression has been well-known to be elevated in endothelial dysfunction (26, 36). Therefore, it is currently held as one of the most reliable biomarkers of endothelial dysfunction/damage. Actually, it has been reported that chronic inhibition of endothelial NO synthesis by L-NAME in rats induces early vascular inflammatory changes, including monocyte infiltration into coronary vessels, monocyte chemoattractant protein-1 expression, and ICAM-1 expression, as well as subsequent atherosclerosis (37, 38). Then we examined the expression levels of ICAM-1 in the aorta by immunohistochemistry. When rats were administered L-NAME for three weeks, increased ICAM-1 expression was observed compared to the control, which was almost fully suppressed to the control levels by nitrosonifedipine treatment. It was reported that long-term L-NAME treatment in rats caused significant reduction of plasma superoxide dismutase activity and following high O₂⁻ production (39, 40). Taking into consideration that O₂⁻ is an important initiator of ICAM-1 expression (28, 41), our findings suggest that nitrosonifedipine may attenuate L-NAME–induced ICAM-1 expression through the suppression of ROS. It has been well known that chronic inhibition of NO synthesis by L-NAME causes renal dysfunction such as the elevation of serum creatinine and urinary protein excretion levels (42, 43). Administration of L-NAME significantly increased serum creatinine levels at week 3 compared to the control group (Table 1). However, it was alleviated by nitrosonifedipine administration in L-NAME–treated rats. In addition, although administration of L-NAME for three weeks decreased creatinine clearance compared to the control group, nitrosonifedipine improved the creatinine clearance reduction (Table 1). Furthermore, administration of L-NAME significantly increased urinary protein excretion per 24 h compared to the control (Table 1). It was also significantly reduced in the L-NAME + nitrosonifedipine group. These findings suggest that nitrosonifedipine has the ability of renoprotection in L-NAME–induced hypertensive rats.

Concluding remarks

In conclusion, in this review we described that 1) ECs convert nitrosonifedipine into nitrosonifedipine radical, which has DPPH radical scavenging activity; 2) nitrosonifedipine shows protective effects against CuOOH- and TNF-α–induced cytotoxicity in ECs; and 3) nitrosonifedipine has EC protective activity and provide renoprotection in L-NAME–induced hypertensive rats. These preliminary data suggest that nitrosonifedipine can be a new and useful drug for the treatment of CKD involving ameliorating effects against EC disorder. Further studies to elucidate the precise mechanisms by which nitrosonifedipine exerts potential therapeutic effects on CKD via the protection of endothelial function are currently under way.

References


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Table 1. Physical findings and laboratory data at the end of 3-week treatment period

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<thead>
<tr>
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<th>Control</th>
<th>L-NAME</th>
<th>L-NAME + nitrosonifedipine</th>
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<tbody>
<tr>
<td>Systolic blood pressure</td>
<td>107 ± 3</td>
<td>146 ± 7*</td>
<td>130 ± 2</td>
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<tr>
<td>(mmHg)</td>
<td></td>
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<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.49 ± 0.04</td>
<td>0.74 ± 0.04*</td>
<td>0.47 ± 0.06†</td>
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<tr>
<td>Creatinine clearance</td>
<td>7.6 ± 1.2</td>
<td>2.4 ± 0.3*</td>
<td>9.2 ± 1.6†</td>
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<tr>
<td>(ml/min/kg)</td>
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<tr>
<td>Urinary protein excretion</td>
<td>30 ± 4</td>
<td>49 ± 4*</td>
<td>33 ± 3*</td>
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<td>(mg/24h)</td>
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Data are means ± S.E.M. *P<0.05 versus Control, †P<0.05 versus L-NAME. L-NAME indicates N⁴-nitro-L-arginine methyl ester. The dose of nitrosonifedipine was 30 mg/kg per day.


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