

ORIGINAL**Protective effect of photodegradation product of nifedipine against tumor necrosis factor alpha-induced oxidative stress in human glomerular endothelial cells**

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Abstract : Recently, increasing evidence suggests that the antihypertensive drug nifedipine acts as a protective agent for endothelial cells, and that the activity is unrelated to its calcium channel blocking. Nitrosonifedipine (NO-NIF) is metabolically and photochemically produced from nifedipine, and NO-NIF has been recognized as a contaminant of nifedipine because it has no antihypertensive effect. Treatment of tumor necrosis factor- α (TNF- α) suppressed the cell viability and facilitated the expression of Inter-Cellular Adhesion Molecule 1(ICAM-1) in human glomerular endothelial cells (HGECs) though, pretreatment of NO-NIF significantly recovered the TNF- α -induced cell damage to the same extent as Trolox-C did, and suppressed the ICAM-1 expression in a concentration dependent manner. In addition, NO-NIF inhibited the cell toxicity induced by cumene hydroperoxide, which hampers the integrity of cell membrane through oxidative stress, as effective as Trolox-c. These data suggest that NO-NIF is a candidate for a new class of antioxidative drug that protect cells against oxidative stress in glomerular endothelial cells. *J. Med. Invest.* 58 : 118-126, February, 2011

Keywords : nifedipine, nitrosonifedipine, antioxidant, tumor necrosis factor- α , reactive oxygen species, endothelial cells, cumene hydroperoxide

INTRODUCTION

Calcium channel blockers have been used for more than 30 years to treat a variety of cardiovascular diseases including angina, arrhythmias and hypertension (1). Nifedipine [1,4-dihydro-2,6-dimethyl-

4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester] is a 1,4-dihydropyridine-derivative calcium channel blocker widely used for treatment of hypertension and angina. Over the past decade, many clinical studies have suggested that nifedipine has not only hypotensive effects but also cardiovascular organ and kidney-protective effects in patients with cardiovascular and kidney diseases (1-4, 5). In addition, it has been reported that nifedipine ameliorates endothelial dysfunctions (6-10) through an anti-apoptotic effect (11, 12) in human endothelial cells. However, it is unlikely that nifedipine exerts

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these effects through its calcium channel-blocking property because endothelial cells do not have voltage-dependent L-type calcium channels, which are thought to be the target of 1,4-dihydropyridine derivatives (13). In addition, nifedipine exerts a direct effect on endothelial cell permeability by inhibiting protein kinase C and attenuates Nuclear Factor-KappaB (NF- κ B) activation, which are independent of its calcium channel-blocking activity (14, 15). These observations suggest that nifedipine has organ protective effects including renoprotection, at least in part, beyond its antihypertensive actions. However its detailed mechanisms have not been identified as yet. Some researchers claimed that the antioxidant activity may be one possible mechanism responsible for the organ-protective effects of 1,4-dihydropyridine calcium channel blockers (16-18). However, the antioxidant activity of nifedipine is reported to be less prominent than that of other 1,4-dihydropyridine calcium channel blockers (16, 17).

Nifedipine is extremely sensitive to light and can be converted to its nitroso analog, nitrosonifedipine [2,6-dimethyl-4-(2-nitrosophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester] (NO-NIF), under normal room illumination (19-25) (Fig. 1). On the other hand, NO-NIF is also enzymatically produced from nifedipine without exposure to light (26). The ability of NO-NIF to block calcium channels is quite weak or non-existent compared with that of nifedipine (27-29). Meanwhile, it was reported that NO-NIF showed a prominent free-radical scavenging activity for 2,2'-azo-bis(2-amidinopropane)-derived alkylperoxyl radicals than that of Trolox-C, a water-soluble vitamin E analogue (30). In the previous report, we had demonstrated that NO-NIF changed to a NO-NIF radical when it interacted with unsaturated fatty acids, a major component of lipid bilayer, and the NO-NIF radicals responsible for

the radical scavenging activity and following prevention of lipid peroxidation (31). Glomerular endothelium is a fundamental layer in the glomerulus in order to protect the glomerulus from atherogenic insults (32). Therefore, dysfunction of glomerular endothelial cells is responsible for the renal diseases progression such as chronic kidney disease (CKD) (33, 34). These observations have led us to hypothesize that NO-NIF would be a critical molecule in the renal glomerular protective effects of nifedipine. Meanwhile, up-regulation of tumor necrosis factor- α (TNF- α) is accomplished by inflammation reactions (35), and associated with the development or exacerbation of kidney diseases such as nephrotic syndrome (36), septic acute renal failure (37), renal ischemia-reperfusion injury (38), cisplatin-induced nephrotoxicity (39), and type 1 diabetes (40). Therefore, in this study, we investigated the ability of NO-NIF to protect endothelial cells from cumene hydroperoxide (Cum-OOH)-induced inflammation (35) and TNF- α induced cell toxicity using cultured human glomerular endothelial cells (HGECs).

MATERIALS AND METHODS

Materials

Nifedipine, methanol, and cumene hydroperoxide (Cum-OOH) were purchased from Wako Pure Chemical Industries (Tokyo, Japan). 6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox-C) was obtained from Tokyo Kasei Co. (Tokyo, Japan). TNF- α (human, recombinant) was from Roche Diagnostics (Germany). Anti intracellular adhesion molecule-1 (anti ICAM-1) antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). NO-NIF was prepared from nifedipine according to a previous report (31). All other reagents were of analytical grade. Water was demineralized and further purified using a Milli-Q system from Millipore (Bedford, MA, USA).

Cell culture

HGECs and CS-C complete medium (including 10% serum) or serum-free CS-C medium were purchased from the Cell Systems (Kirkland, WA, USA). HGECs were cultured at a density of 1×10^5 cells/ml in 60 mm dishes or 24-well plates (Iwaki Glass Co., Ltd.) in CS-C complete medium at 37°C in an incubator containing 5% CO₂ and used within five passages.

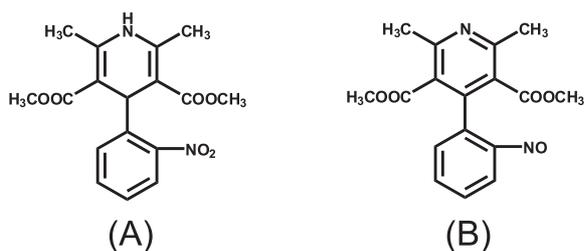


Figure 1. Chemical structure of nifedipine (A) and NO-NIF (B).

Cell viability assay

The cell damage induced by Cum-OOH and TNF- α and its attenuation by NO-NIF was evaluated by assessing the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) as previously described (41). Briefly, 90% confluent HGECs in 24-well culture plates were washed twice with phosphate buffered saline (PBS) then incubated with or without test reagent in CS-C complete medium for 6-h. Following 24-h incubation with TNF- α or with Cum-OOH, culture medium was sucked out then added 500 μ L of MTT-containing serum free CS-C medium and after a further 3-h incubation. HGECs were lysed with 10% SDS solution containing 0.01N HCl, and the amount of formozan was assessed by measuring the absorption at 590-nm using a spectrophotometer as an index of the cell viability. The cell viability (%) was calculated using the following formula :

$$\text{Cell viability (\%)} = 100 \times \text{A}_{590} (\text{sample}) / \text{A}_{590} (\text{negative control})$$

Western blotting analysis of ICAM-1

HGECs were grown to 90% confluency on 60 mm dish with CS-C complete medium then treated with TNF- α at indicated time and concentration in each figure legend. Thereafter, the incubation media were discarded, washed twice with ice-cold Ca²⁺- and Mg²⁺-containing PBS and lysed in 500 μ L of lysis buffer (20 mM Tris-HCl, pH7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycero-phosphate, 1 mM sodium orthovanadate, 1 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). After subjecting the cells to a freeze-thaw cycle, the cell lysates were transferred to micro-centrifuge tubes, sonicated (Handy Sonic UR-20 P; Tomy Seiko Co., Ltd.) on ice, and then centrifuged at 20,000 xg for 20 min at 4°C. The protein amounts in the cell extracts were determined using a protein assay kit (BIO-RAD, Hercules, CA), and the extracts were then stored at -80°C until use.

Western blotting analysis of ICAM-1 protein expression in HGECs was carried out using an ICAM-1-specific antibody. Briefly, the cell extracts (20 μ g of total protein per lane) were analyzed by SDS-PAGE on a 10% polyacrylamide gel, and proteins were transferred onto a PVDF membrane and the blots were first blocked with 5% bovine serum albumin for 3-h. Subsequently, the membrane was

incubated with a polyclonal rabbit anti-ICAM-1 antibody (1 : 2000 dilution) at room temperature for 1-h, and the immunocomplex of ICAM-1 protein was then visualized followed by incubation with ECL western-blotting kit and Hyperfilm ECL (GE healthcare, Piscataway, NJ, USA) in accordance with the manufacturer's instructions. The signal intensities of corresponding bands were determined by a computerized densitometric analysis using Image-J software.

Statistical analysis

Values are expressed as means \pm S.D. for 3-6 separate experiments. Data were analyzed using a one-way analysis of variance was used to determine significance among groups, after which a modified *t*-test with the Bonferroni correction for comparison between groups. Values of $p < 0.05$ were accepted as statistically significant.

RESULTS

Effect of NO-NIF on Cum-OOH-induced cytotoxicity

Since Cum-OOH is a lipid soluble hydroperoxide and known to induce lipid peroxidation and apoptotic cell damage (42) through free radical reaction (43), we investigated the HGECs viability following exposure to Cum-OOH. 24-h incubation of Cum-OOH significantly and dose dependently decreased cell viability in HGECs (Fig. 2). To investigate the antioxidant activity of NO-NIF to the Cum-OOH, HGECs were pretreated with 10 μ M NO-NIF

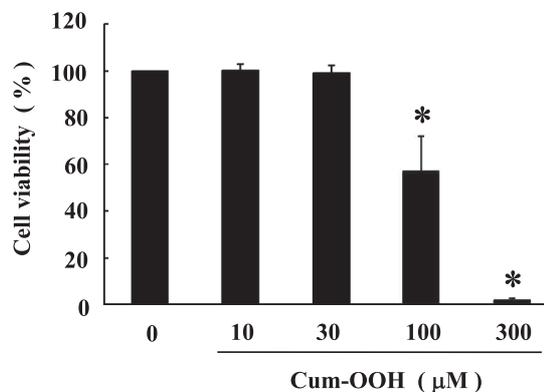


Figure 2. Effect of Cum-OOH on viability of HGECs. HGECs were exposed to various concentrations of Cum-OOH for 24 h. Thereafter the cell viability was examined using a MTT assay as described in Materials and Methods. Results were expressed as a percentage of control. Values are the mean \pm SD (* $p < 0.05$ compared with control, $n = 6$).

for 6-h and following 24-h incubation in the presence of 100 μ M Cum-OOH. As shown in Fig. 3, the cell viability was significantly recovered by the addition of NO-NIF (90 \pm 9% of control), which was to the same extent as Trolox-C (80 \pm 5% of control) but not by nifedipine (64 \pm 14% of control).

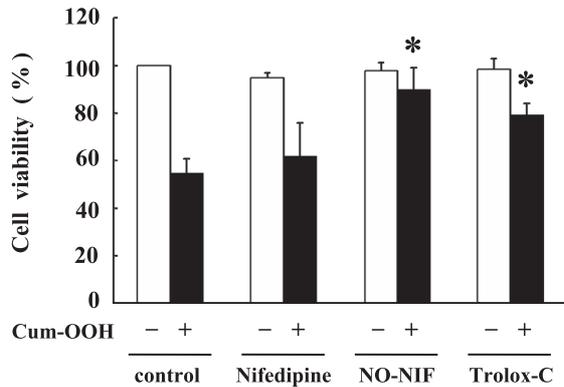


Figure 3. Protective effect of NO-nif on Cum-OOH-induced damage to HGECs. HGECs were preincubated with 10 μ M of each reagent (NO-NIF, nifedipine, and Trolox-C) for 6 h, and then exposed to 100 μ M Cum-OOH for 24 h. The cell viability were then assessed using a MTT assay as described in Materials and Methods. Results were expressed as a percentage of those obtained from the group of Cum-OOH alone. Values are the mean \pm SD (* p <0.05 compared with Cum-OOH(+) control, n = 6).

Effect of NO-NIF on TNF- α -induced cytotoxicity

Firstly, to assess the cytotoxic effect of TNF- α on HGECs, the cells were exposed to various concentrations of TNF- α for 24h, and the cell viability was then determined by analyzing the reduction of MTT to formazone as an index of the viable cells. As shown in Fig. 4, TNF- α reduced the number of viable cells in a dose-dependent manner, and the approximately 90.6 \pm 6.4%, 78.0 \pm 2.3% and 62.7 \pm 11% reduction of the cell viability was observed at the concentrations of 1, 3 and 10 ng/ml, respectively. Then, to examine the cytoprotective effect of NO-NIF, the HGECs were pretreated with 10 μ M NO-NIF for 6-h following 24-h treatment with 10 ng/ml TNF- α , and the cell viability was determined by measuring MTT reduction under the same experimental conditions. As shown in Fig. 5, the reduction of cell viability induced by TNF- α was completely blocked by pretreatment of the cells with NO-NIF, but not with nifedipine. In addition, Trolox C, a water-soluble derivative of vitamin E with antioxidant activity, also completely protected the cells against the cytotoxic effect of TNF- α to

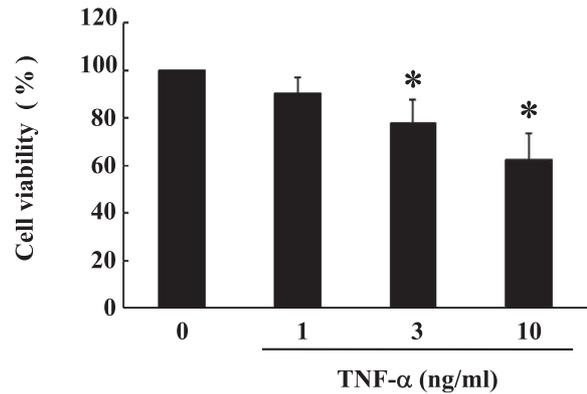


Figure 4. Effect of TNF- α on viability of HGECs. HGECs were exposed to various concentrations of TNF- α for 24 h. Thereafter the cell viability was examined using a MTT assay as described in Materials and Methods. Results were expressed as a percentage of control. Values are the mean \pm SD (* p <0.05 compared with control, n = 5).

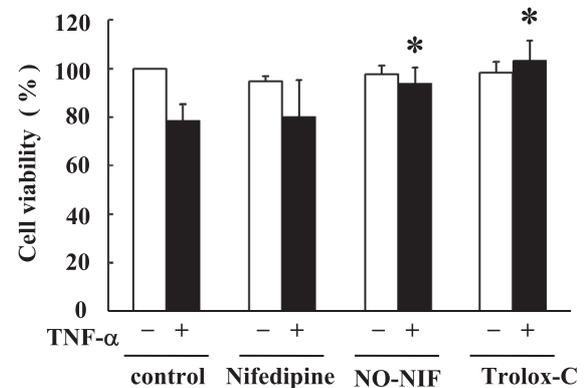


Figure 5. Protective effect of NO-NIF on TNF- α -induced damage to HGECs. HGECs were preincubated with 10 μ M of each reagent (NO-NIF, nifedipine, and Trolox-C) for 6-h, and then exposed to 10 ng/mL TNF- α for 24-h. The cell viability was then assessed using a MTT assay as described in Materials and Methods. Results were expressed as a percentage of those obtained from the group of TNF- α alone. Values are the mean \pm SD (* p <0.05 compared with TNF- α (+) control, n = 5).

the same extent as NO-NIF.

TNF- α induced ICAM-1 protein expression in HGECs

To examine the effect of TNF- α on ICAM-1 protein expression in HGECs, the cells were treated with various concentrations of TNF- α for different time periods, and the amounts of ICAM-1 protein in the cells were determined by a immunoblotting technique. As shown in Fig. 6, TNF- α increased ICAM-1 protein expression in HGECs in a manner depending upon the time and concentration of TNF- α treatment. The significant elevation of ICAM-1 protein expression was observed by exposing the cells to 10 ng/ml of TNF- α for at least 6 h, and

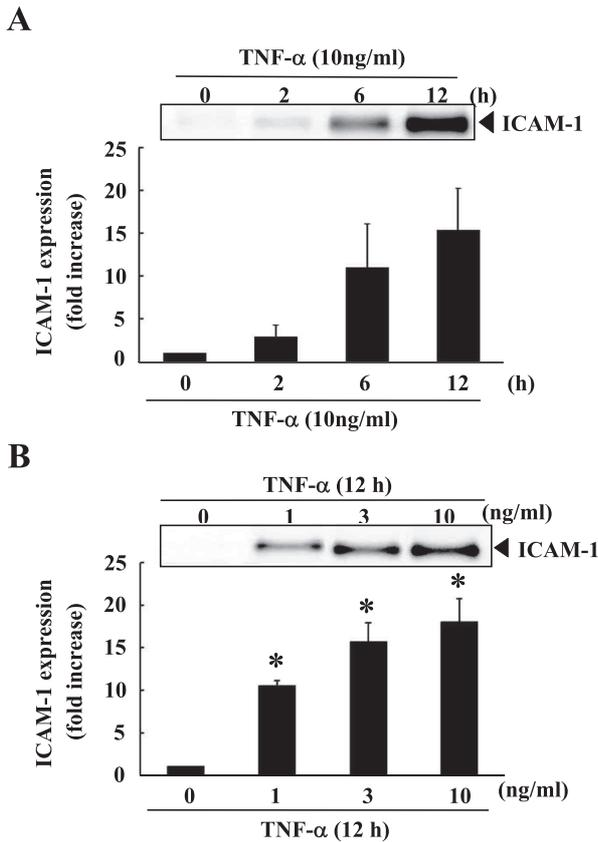


Figure 6. Time- and concentration-dependent effect of TNF- α on ICAM-1 protein expression in HGECs. HGECs were exposed to 10 ng/ml TNF- α for different time periods (A), or incubated with various concentrations of TNF- α for 12 h (B). Cell extracts were prepared and subjected to an immunoblotting analysis as described in Materials and Methods, and the expression of ICAM-1 protein was then assessed by determining the intensities of corresponding bands. Results were normalized to the control, and expressed as a fold-increase. Values are the mean \pm SD (* p <0.05 compared with non-stimulated HGECs, $n = 4$).

also induced by exposing to the considerably low concentration of TNF- α (1 ng/ml) for 12-h.

Effect of NO-NIF on TNF- α -induced ICAM-1 protein expression in HGECs

The effect of NO-NIF on TNF- α -induced ICAM-1 protein expression in HGECs was examined to elucidate a possible relation between TNF- α -induced ICAM-1 protein expression and its toxic effect on the cells. As shown in Fig. 7, TNF- α -induced ICAM-1 protein expression was considerably reduced by the pretreatment of the cells with NO-NIF in a concentration dependent manner. In contrast, nifedipine had no effect on TNF- α -induced ICAM-1 protein expression even at the highest concentration used here.

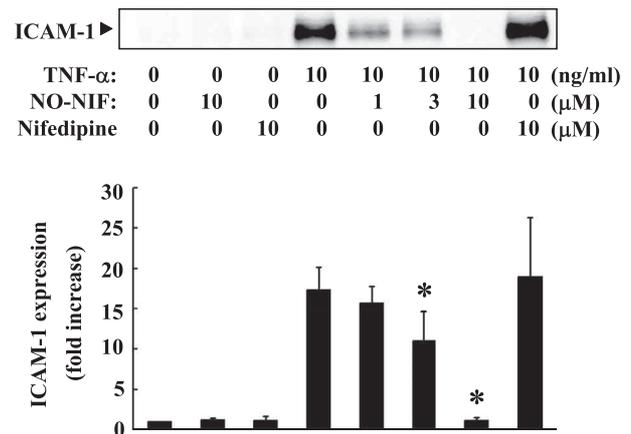


Figure 7. Effect of NO-NIF and nifedipine on TNF- α -induced ICAM-1 expression in HGEC.

HGECs were pretreated with various concentrations of NO-NIF or nifedipine for 6 h, and then exposed to 10 ng/ml of TNF- α for 12 h. Cell extracts were prepared and subjected to an immunoblotting analysis as described in Materials and Methods, and the expression of ICAM-1 protein was then assessed by determining the intensities of corresponding bands. Results were normalized to the control, and expressed as a fold-increase. Values are the mean \pm SD (* p <0.05 compared with TNF- α (10 ng/ml), $n = 5$).

DISCUSSION

Nifedipine is a dihydropyridine (DHP)-based L-type calcium channel antagonists (44), and it has been used for the treatment of hypertension and angina pectoris since the 1960s. In addition to its hypotensive and anti-anginal effects, pleiotropic effects of nifedipine were recently reported: anti-apoptotic, anti-inflammatory, and antioxidant effects on endothelial cells that control the development and progression of atherosclerosis (2, 7-12, 15, 45-48).

Endothelium is a fundamental layer in the arterial wall for the local regulation of flow to organ like the kidney, and the injury of it can be a critical insult for the generation and progression of CKD (32, 34). Therefore, nifedipine has been predicted to be a candidate for treatment of CKD, but finds equivocal effects on the progression of CKD from the results of clinical trials including ALLHAT (The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial) and INSIGHT (Intervention as a Goal in Hypertension Treatment) except in the case of adequate control of blood pressure is achieved by nifedipine (44). Furthermore, it was reported that the treatment of CKD by nifedipine is likely to worsen rather than improve due to the development of glomerular hypertension caused

by its L-type channel blocking activity (44).

By the way, nifedipine is light-sensitive, and is converted completely to its nitroso analog, NO-NIF (Fig. 1) without further photochemical degradation under ordinary light (26). In addition, it has been reported that NO-NIF lost the L-type Ca^{2+} -channel blocking activity (29). To date, it has been reported that NO-NIF reduced the alkylperoxyl radicals on its nitroso aromatic group, the kinetic rate constant was ten and two times higher than that of nifedipine and Trolox-C (30), respectively, NO-NIF reacted with unsaturated fatty acids to form nitroxide radicals *via* a pseudo-Diels-Alder mechanism (21, 49), and the NO-NIF radical played an essential role for antioxidative activity of NO-NIF (31).

These observations led us to hypothesize that the NO-NIF can be a candidate for glomerular endothelial protecting agent for CKD patients if it possesses anti-inflammatory activity. Therefore in the present study, we investigated the ability of the NO-NIF to protect cells from inflammation-derived insult induced by Cum-OOH and TNF- α using HGECs.

When HGECs were stimulated by 100 μM Cum-OOH, cell viability was significantly decreased and the Trolox-C treatment almost completely recovered, indicating that oxidative modulation of cell membrane participated in the toxicity of Cum-OOH as described previously (35, 50). The addition of 10 μM NO-NIF improved the cell viability either equaling or suppressing the that of Trolox-C whereas nifedipine did not (Fig. 3), and these results were in good agreement with the order of radical scavenging activity as reported (30). These data suggested that NO-NIF improves the cytotoxicity based on its quenching activity against lipid peroxides as demonstrated in cell-free system (31).

It is apparent that the up-regulation of TNF- α is closely related to the development or exacerbation of kidney diseases (36-40). As shown in Fig. 4, TNF- α treatment showed cytotoxicity as reported (51). Because TNF- α increases cellular oxidation (52), which is responsible for cell damage. Therefore, it was expected that antioxidative activity of NO-NIF (31) contributes to the suppression of TNF- α -induced cell damages, and that the NO-NIF could suppress the expression of cell adhesion molecules, because TNF- α stimulates expression of cell adhesion molecules such as ICAM-1 through its inflammation reaction activities (53-57). Indeed, ICAM-1 expression was augmented by TNF- α treatment in a time and concentration dependent manner in

HGECs (Fig. 6), and 10 μM NO-NIF treatment significantly diminished the expression of ICAM-1 induced by TNF- α ($8 \pm 1\%$ of TNF- α treatment), but nifedipine showed no effects (10 μM , $102 \pm 2\%$ of TNF- α treatment) (Fig. 7). Furthermore, we had demonstrated that NO-NIF suppressed the production of superoxide anion radical, a kind of reactive oxygen species, induced by LY83583 (31). Taking this evidence together with our present study, the suppression of TNF- α -induced ICAM-1 expression by NO-NIF is suggested to be involved in its ROS scavenging ability in HGECs.

In conclusion, we found that NO-NIF shows protective effects against Cum-OOH- and TNF- α -induced cytotoxicity in HGECs through its antioxidative properties (31). Because NO-NIF is one of the metabolites of nifedipine (26) and its intrinsic toxicity was equal to that of nifedipine in HGECs (Figs 3 and 5), we expect that NO-NIF can be a candidate for an antioxidant drug to protect glomerular endothelial cells against TNF- α -related diseases.

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