Nafamostat mesilate promotes endothelium-dependent vasorelaxation via the Akt-eNOS dependent pathway

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INTRODUCTION

Vascular endothelial cells are critically implicated in the modulation of vascular tone through the production of a variety of vasoactive substances. This modulation is known to be impaired in pathophysiological states such as diabetes mellitus, hypertension, and hyperlipidemia [1]. Nitric oxide (NO) is a major endothelium-dependent relaxing factor, and its production by vascular endothelial cells plays a vital role in the regulation of vascular motor tone and stability of blood flow as well as blood pressure [2,3]. Endothelial nitric oxide synthase (eNOS), one of three NOS isoforms produces NO, which has a key role in regulating systemic blood pressure [4]. Previous reports have shown that a decrease in NO production can lead to hypertension [5], and eNOS mutation leads to impaired endothelium-dependent vasorelaxation and may have hypertensive effects [6]. Taken together, NO produced by eNOS is a fundamental determinant of cardiovascular homeostasis, and regulates systemic blood pressure, vascular remodeling and angiogenesis. Furthermore, it has been reported that eNOS is activated by direct phosphorylation of serine 1179 (Ser1179) by Akt, a downstream target of phosphatidylinositol 3-kinase [7]. NO

ABSTRACT Nafamostat mesilate (NM), a synthetic serine protease inhibitor, has anticoagulant and anti-inflammatory properties. The intracellular mediator and external anti-inflammatory external signal in the vascular wall have been reported to protect endothelial cells, in part due to nitric oxide (NO) production. This study was designed to examine whether NM exhibit endothelium dependent vascular relaxation through Akt/endothelial nitric oxide synthase (eNOS) activation and generation of NO. NM enhanced Akt/eNOS phosphorylation and NO production in a dose- and time-dependent manner in human umbilical vein endothelial cells (HUVECs) and aorta tissues obtained from rats treated with various concentrations of NM. NM concomitantly decreased arginase activity, which could increase the available arginine substrate for NO production. Moreover, we investigated whether NM increased NO bioavailability and decreased aortic relaxation response to an eNOS inhibitor in the aorta. These results suggest that NM increases NO generation via the Akt/eNOS signaling pathway, leading to endothelium-dependent vascular relaxation. Therefore, the vasorelaxing action of NM may contribute to the regulation of cardiovascular function.
is produced by vascular endothelial cells using L-arginine as a substrate in a process catalyzed by NOS [8] and induces vascular smooth muscle relaxation by activation of guanylate cyclase [8,9]. Inhibition of eNOS with Nω-nitro-L-arginine methyl ester (L-NAME), resulted in significantly enhanced blood pressure due to decreased NO production [10]. Recent studies suggest that production of NO is reduced and endothelium-dependent vasorelaxation is blunted in patients with essential hypertension [11].

Nafamostat mesilate (NM), a serine protease inhibitor, is used to treat patients suffering from disseminative blood vessel coagulation, hemorrhagic lesions, and hemorrhagic tendencies. NM also improves acute pancreatitis and prevents blood clots formation during extracorporeal circulation [12,13]. Our previous studies have indicated that NM inhibits tumor necrosis factor alpha (TNF-α)-induced vascular endothelial cell dysfunction [14], and attenuates transient focal ischemia/reperfusion-induced brain injury [15]. Recent reports suggest that the anti-inflammatory mechanisms in the vascular wall, which include external anti-inflammatory signals and intracellular mediators, protect endothelial cells, in part due to NO production [16,17]. Moreover, there is a diverse range of serine protease inhibitors, including synthetic chemical inhibitors, and natural proteinaceous inhibitors, available for research or therapeutic purposes. Szabo et al. reported that synthetic serine protease inhibitors have an effect on vascular relaxation after cardiac surgery [18]. Chao et al. reported that kallistatin, a natural serine protease inhibitor, is a potent vasodilator which operates via a vascular smooth muscle mechanism [19]. However, the study was limited to the detection of vascular contraction and did not investigate the associated regulation mechanisms and signaling pathways. NM, as another serine protease inhibitor, may possess a similar vasodilator ability.

Therefore, in this study, we addressed this hypothesis by studying the effect of NM on NO production, NO bioavailability and vascular relaxation in vivo and NO production, arginase activity in vitro; mediated through the Akt/eNOS phosphorylation dependent signaling pathway.

**METHODS**

**Cell culture**

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA, USA) and cultured in endothelial growth medium (EGM-2). Sub-confluent, proliferating HUVECs at passages 2–8 were used.

**Western blot analysis**

Anti-phospho-eNOS antibody was purchased from Cell Signaling (Beverly, MA, USA). Anti-NOS3, anti-β-actin, anti-phospho-Akt and total Akt antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Western blot analysis was performed by boiling 30 μg of whole cell lysate or 30 μg of tissue homogenate (obtained from rat aorta) in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer, before separation by electrophoresis and transfer to a nitrocellulose membrane. After incubation in appropriate primary and peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), chemiluminescent signaling was developed using Super Signal West Pico or Femto Substrate from Thermo Fisher Scientific (Pierce, Rockford, IL, USA). Blots were imaged and band densities quantified with a Gel Doc 2000 Chemi Doc system using Quantity One software from Bio-Rad (Hercules, CA, USA). Values were normalized to a β-actin loading control.

**Animals**

The present study utilized 6 week old male Sprague–Dawley (SD) rats (Samtako, Osan, Korea) with body weights of between 250 and 280 g. All experimental procedures adhered to the policies of Chungnam National University regarding the use and care of animals. All animals were housed in a standard environment with a 12:12 h light/dark cycle, a constant room temperature maintained at 20–25°C, and 40–60% humidity. Food and water were supplied ad libitum.

**Nitrite and nitrate measurements**

Two NO metabolites, nitrite (NO$\cdot^{2-}$) and nitrate (NO$\cdot^{3-}$), the stable breakdown products of NO, were quantified using a commercially available Nitrate/Nitrite Fluorometric Assay Kit from Cayman Chemicals (Lexington, KY, USA), as per the manufacturer’s instructions. Plasma obtained from the rat blood was deproteinized using a 10 kDa cutoff filter (Microcon YM-10, Millipore, USA) and used for the quantification of NO.

**DAF-FM DA staining**

DAF-FM DA is a cell-permeable fluorescent probe for the detection of NO. This was chosen in preference to the widely used DAF-2 as DAF-FM and DAF-FM DA are more sensitive to NO, more photo-stable, and less pH sensitive. DAF-FM DA permeates living cells and is rapidly transformed into water-soluble DAF-FM by cytosolic esterases. Aortic sections stained with DAF-FM DA were imaged using a fluorescence microscope. All images from control and NM treated rings were captured using identical laser intensity, brightness, and contrast settings.

**Arginase activity assay**

Arginase activity was measured using Quanti Chrom Arginase...
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Vascular reactivity

Rats were sacrificed via sodium pentobarbital overdose. A mid-sternal split was quickly performed, and the descending thoracic aorta was carefully excised and placed in ice-cold Krebs buffer (118.3 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 1.2 mM MgSO₄, 11 mM glucose, and 0.0026 mM EDTA-CaNa₂). The aorta was cleaned of excess fat, cut transversely into 5–10 rings (2.0–3.0 mm), and maintained at 37°C and pH 7.4. Endothelium-dependent vasodilation was determined by generating dose–response curves in aortic rings pre-constricted with phenylephrine.

Statistical analysis

All experiments were performed at least three times. All data are expressed as means±standard deviations. Statistical analysis was performed using Sigma Stat (Systat Software, La Jolla, CA, USA). Data in which two conditions were compared were evaluated using one-way analysis of variance followed by Tukey’s post-hoc test; p-values<0.05 were considered significant.

RESULTS

NM stimulates p-eNOS and p-Akt expression in HUVECs

We investigated the effect of NM on the expressions of p-endothelial cell nitric oxide synthase (p-eNOS) and p-Akt in endothelial cells. For the dose-dependent experiment, endothelial cells were treated with different doses of NM for 2h and harvested for western blot analysis. For the time-dependent experiment, HUVECs were treated with 300 ng/ml of NM for different time periods from 5 min to 2 h before analysis by western blot. As shown in Fig. 1A and 1D, phosphorylation of eNOS and Akt increased in both dose- and time-dependent manner, compared to the total forms of the respective proteins. These results suggest that NM stimulates p-eNOS and p-Akt expression in endothelial cells.

NM stimulates the production of NO in HUVECs

NO is an endothelium derived relaxing factor, which plays an important role in the control of vascular tone and function. Phosphorylation of eNOS and Akt leads to stimulation of NO production in endothelial cells. We therefore measured the level of NO produced by HUVECs treated with different doses of NM.

![Fig. 1. Nafamostat mesilate (NM) dose- and time-dependently stimulates phosphorylation of endothelial nitric oxide synthase (eNOS) and Akt in human umbilical vein endothelial cells (HUVECs).](image-url)
Fig. 2A shows that NM treatment dose-dependently increased the production of NO in HUVECs. NO is produced from L-arginine by the activity of eNOS. L-arginine is a common substrate for NOS, metabolized by arginase to produce urea and ornithine. We measured the arginase activity in HUVECs treated with NM and found that there was a subsequent dose-dependent decrease in arginase activity (Fig. 2B), indicating increased eNOS activity after NM treatment.

NM stimulates p-eNOS and p-Akt expression ex vivo

To identify the physiological relevance of NM on eNOS and vascular function, we first examined whether the phosphorylation of NOS and Akt in aortic rings treated with NM was activated. Aortas from SD rats were obtained, cut into segments and incubated with various doses of NM for 2 h, then analyzed by western blot analysis. As shown in Fig. 3A, phosphorylation of both eNOS and Akt increased with increasing dose of NM, similar to the in vitro results.
NM stimulates the production of nitric oxide ex vivo

Next, we examined the effect of NM on NO production in aortic rings. Aortas from SD rats were obtained, cut into segments and incubated with various doses of NM for 2 h followed by incubation with DAF dye for 15 min in the dark. The aortic rings were then sliced and mounted on a glass slide for analysis by microscopy. As shown in Fig. 3D, NM treatment dose dependently increased the production of NO, as evidenced by increasing DAF fluorescence intensity which is consistent with the in vitro results. We also measured NO levels in the plasma obtained from the blood of rats treated with different dose of NM. Increased dose of NM stimulated the production of NO (Fig. 3F).

NM improves impaired endothelial-dependent vascular relaxation ex vivo

Finally, we tested whether NM treatment stimulated vascular reactivity. Fig. 4A shows that NM treatment in aortic rings preconstricted with phenylephrine (10^{-5} M) dose-dependently induced an increase in vasorelaxation. However, NM treatment did not induce as much vasorelaxation in L-NAME (which is a NOS inhibitor) pretreated aortic rings suggesting the importance of eNOS activation in the development of relaxation of vessels. At the same time, bioavailability of NO was higher in NM treated aortic rings compared to the saline vehicle control (Fig. 4B) further supporting the concept of eNOS activation. These data collectively suggest that vascular function, as well as eNOS activity, is improved with NM treatment of vascular endothelial cells.

DISCUSSION

In the present study, we investigated the effect of NM on NO production and vascular relaxation mediated by phosphorylation of Akt and eNOS and found that NM stimulated Akt/eNOS phosphorylation and NO production in a dose- and time-dependent manner (Fig. 4C). NM also caused a decrease in arginase activity. The NM-induced decreases in arginase activity could improve the available arginine substrate for NO production. We also detected that the aortic relaxation was significantly decreased when adding L-NAME to inhibit eNOS phosphorylation. These results suggest that NM promotes endothelium-dependent vasorelaxation by enhancing Akt and eNOS phosphorylation and that NM administration has the potential to stabilize blood pressure and may be effective to prevent cardiovascular diseases.

We previously showed that NM produces anti-inflammatory effects on vascular endothelial cells [14], and neuroprotective effects in the injured brain by inhibiting endoplasmic reticulum stress [15]. As previously reported, anti-inflammatory mechanisms protect endothelial cells partly through NO production [17,19], which may be effective in NO-induced vasodilation. In the present study, relaxation of aortic rings was observed in the group which was administered NM (Fig. 4A). Subsequently, we investigated the signaling pathway responsible for vascular relaxation response to NM.

NO, the endothelium-derived vasoactive factor, plays an important role in modulating vasodilation. eNOS, also known as nitric oxide synthase 3, generates NO in blood vessels and is involved in the regulation of vascular function. Recent reports have suggested that in response to a variety of stimuli, efficient NO production requires eNOS phosphorylation via the Akt pathway [7,20,21]. Therefore, we investigated whether NM activated Akt/eNOS phosphorylation. Previously, we have shown that incubation with various concentrations of NM (0.01–100 ng/ml) for 24 h did not affect the viability of HUVECs [14]. In the present study, our data showed that NM (10–1000 ng/ml) treatment induced Akt and eNOS phosphorylation in a dose- and time-
dependent manner in HUVECs within 2 h (Fig. 1), suggesting that NM-induced Akt phosphorylation mediates the activation of eNOS, and may lead to increased NO production. Next, we measured the downstream target of Akt/eNOS phosphorylation that is NO production (Fig. 2). Another possibility is that NM induces vasorelaxation directly, not via activation of Akt/eNOS pathway and subsequent increased NO production. In order to clarify the mechanism, we added L-NAME to inhibit eNOS activity. The aortic relaxation was significantly decreased (Fig. 4A), demonstrating that NM-induces vasorelaxation fully, or in part, via eNOS-mediated NO production. NO is produced from L-arginine by eNOS via the L-citrulline/L-arginine recycling pathway [22]. The enzyme arginase metabolizes L-arginine to L-ornithine and urea. Increasing evidence suggests that arginase upregulation functionally inhibits available arginine substrate for NOS activity and contributes to decreased NO generation [23,24], which means that decreased arginase activity increases L-arginine levels, the common substrate for NOS. Therefore, NM-induced depressed arginase activity observed in this study may promote NO production by releasing more available arginine substrate (Fig. 2B).

Previous studies have demonstrated that NM stimulates Akt/eNOS phosphorylation and NO production in a dose- and time-dependent manner in HUVECs. To examine more specifically the role of NM on endothelium-dependent vasodilation, we detected Akt/eNOS phosphorylation and NO production in rat aortic tissues. Our data showed that NM increased Akt/eNOS expression in a dose-dependent manner in rat aortic tissues (Fig. 3A), as well as NO production in plasma (Fig. 3F). We also observed a significant amount of Akt and eNOS phosphorylation in aortas stimulated with high concentration of NM, nearly 100 times the concentration required in HUVECs. We have clearly observed that NM evoked a conclusive increase in NO-related fluorescence of aorta tissues stained with the fluorescent probe DAF-FM-DA (Fig. 3D). Therefore, it is now clear that NM is fluorescence of aorta tissues stained with the fluorescent probe and may lead to increased NO production. Next, we measured the downstream target of Akt/eNOS phosphorylation that is NO production (Fig. 2). Another possibility is that NM induces vasorelaxation directly, not via activation of Akt/eNOS pathway and subsequent increased NO production. In order to clarify the mechanism, we added L-NAME to inhibit eNOS activity. The aortic relaxation was significantly decreased (Fig. 4A), demonstrating that NM-induces vasorelaxation fully, or in part, via eNOS-mediated NO production. NO is produced from L-arginine by eNOS via the L-citrulline/L-arginine recycling pathway [22]. The enzyme arginase metabolizes L-arginine to L-ornithine and urea. Increasing evidence suggests that arginase upregulation functionally inhibits available arginine substrate for NOS activity and contributes to decreased NO generation [23,24], which means that decreased arginase activity increases L-arginine levels, the common substrate for NOS. Therefore, NM-induced depressed arginase activity observed in this study may promote NO production by releasing more available arginine substrate (Fig. 2B).

In summary, NM stimulates endothelium-dependent vascular relaxation. This vascular effect is mediated by increased production of NO, which occurs via the activation of eNOS via Akt/eNOS signaling pathway.

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**CONFLICTS OF INTEREST**

The authors declare no conflicts of interest.

**REFERENCES**

1. Ross R, Glomset JA. The pathogenesis of atherosclerosis (first of two parts). N Engl J Med. 1976;295:369-377.
2. Rees DD, Palmer RM, Moncada S. Role of endothelium-derived nitric oxide in the regulation of blood pressure. Proc Natl Acad Sci U S A. 1989;86:3375-3378.
3. Vallance P, Collier J, Moncada S. Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. Lancet. 1989;2:997-1000.
4. Shesely EG, Maeda N, Kim HS, Desai KM, Krege JH, Laubach VE, Sherman PA, Sessa WC, Smithies O. Elevated blood pressures in mice lacking endothelial nitric oxide synthase. Proc Natl Acad Sci U S A. 1996;93:13176-13181.
5. Watson T, Goon PK, Lip GY. Endothelial progenitor cells, endothelial dysfunction, inflammation, and oxidative stress in hypertension. Antioxid Redox Signal. 2008;10:1079-1088.
6. Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, Fishman MC. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. Nature. 1995;377:239-242.
7. Dimmelwer S, Fleming J, Fislihalter B, Hermann C, Busse R, Zeiber AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature. 1999;399:601-605.
8. Palmer RM, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature. 1988;333:664-666.
9. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature. 1987;327:524-526.
10. Bobadilla NA, Gamba G, Tapia E, García-Torres R, Bolio A, López-Zetina P, Herrera-Acosta J. Role of NO in cyclosporin nephrotoxicity: effects of chronic NO inhibition and NO synthases gene expression. Am J Physiol. 1998;274:F791-798.
11. Panza JA, Casino PR, Kilcoyne CM, Quyyumi AA. Role of endothelium-derived nitric oxide in the abnormal endothelium-dependent vascular relaxation of patients with essential hyperten-
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12. Akizawa T, Koshikawa S, Ota K, Kazama M, Mimura N, Hirasawa Y. Nafamostat mesilate: a regional anticoagulant for hemodialysis in patients at high risk for bleeding. Nephron. 1993;64:376-381.

13. Iwaki M, Ino Y, Motoyoshi A, Ozeki M, Sato T, Kurumi M, Aoyama T. Pharmacological studies of FUT-175, nafamostat mesilate. V. effects on the pancreatic enzymes and experimental acute pancreatitis in rats. Jpn J Pharmacol. 1986;41:155-162.

14. Kang MW, Song HJ, Kang SK, Kim Y, Jung SB, Jee S, Moon JY, Suh KS, Lee SD, Jeon BH, Kim CS. Nafamostat mesilate inhibits TNF-α-induced vascular endothelial cell dysfunction by inhibiting reactive oxygen species production. Korean J Physiol Pharmacol. 2015;19:229-234.

15. Kwon SK, Ahn M, Song HJ, Kang SK, Jung SB, Harsha N, Jee S, Moon JY, Suh KS, Lee SD, Jeon BH, Kim DW, Kim CS. Nafamostat mesilate attenuates transient focal ischemia/reperfusion-induced brain injury via the inhibition of endoplasmic reticulum stress. Brain Res. 2015;1627:12-20.

16. Gonzalez W, Fontaine V, Pueyo ME, Laquay N, Messika-Zeitoun D, Philippe M, Arnal JF, Jacob MP, Michel JB. Molecular plasticity of vascular wall during N(G)-nitro-L-arginine methyl ester-induced hypertension: modulation of proinflammatory signals. Hypertension. 2000;36:103-109.

17. Tedgui A, Mallat Z. Anti-inflammatory mechanisms in the vascular wall. Circ Res. 2000;88:877-887.

18. Szabó G, Veres G, Radovits T, Haider H, Krieger N, Bährle S, Miesel-Gröschel C, Niklisch S, Karck M, van de Locht A. Effects of novel synthetic serine protease inhibitors on postoperative blood loss, coagulation parameters, and vascular relaxation after cardiac surgery. J Thorac Cardiovasc Surg. 2010;139:181-188; discussion 188.

19. Chao J, Stallone JN, Liang YM, Chen LM, Wang DZ, Chao L. Kallistatin is a potent new vasodilator. J Clin Invest. 1997;100:11-17.

20. García-Cardeña G, Fan R, Shah V, Sorrentino R, Cirino G, Papapetropoulos A, Sessa WC. Dynamic activation of endothelial nitric oxide synthase by Hsp90. Nature. 1998;392:821-824.

21. Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature. 1999;399:597-601.

22. Mori M, Gotoh T. Regulation of nitric oxide production by arginine metabolic enzymes. Biochem Biophys Res Commun. 2000;275:715-719.

23. Berkowitz DE, White R, Li D, Minhas KM, Cernetich A, Kim S, Burke S, Shoukas AA, Nyhan D, Champion HC, Hare JM. Arginase reciprocally regulates nitric oxide synthase activity and contributes to endothelial dysfunction in aging blood vessels. Circulation. 2003;108:2000-2006.

24. Ryoo S, Lemmon CA, Soucy KG, Gupta G, White AR, Nyhan D, Shoukas A, Romer LH, Berkowitz DE. Oxidized low-density lipoprotein-dependent endothelial arginase II activation contributes to impaired nitric oxide signaling. Circ Res. 2006;99:951-960.