Neurogranin regulates eNOS function and endothelial activation

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ABSTRACT

Endothelial nitric oxide (NO) is a critical mediator of vascular function and vascular remodeling. NO is produced by endothelial nitric oxide synthase (eNOS), which is activated by calcium (Ca^{2+})-dependent and Ca^{2+}-independent pathways. Here, we report that neurogranin (Ng), which regulates Ca^{2+}-calmodulin (CaM) signaling in the brain, is uniquely expressed in endothelial cells (EC) of human and mouse vasculature, and is also required for eNOS regulation. To test the role of Ng in eNOS activation, Ng knockdown in human aortic endothelial cells (HAEC) was performed using Ng SiRNA along with Ng knockout (Ng $^{-/-}$) in mice. Depletion of Ng expression decreased eNOS activity in HAEC and NO production in mice. We show that Ng expression was decreased by short-term laminar flow and long-them oscillating flow shear stress, and that Ng siRNA with shear stress decreased eNOS expression as well as eNOS phosphorylation at S1177. We further reveled that lack of Ng expression decreases both AKT-dependent eNOS phosphorylation, NF-κB-mediated eNOS expression, and promotes endothelial activation. Our findings also indicate that Ng modulates Ca^{2+}-dependent calcineurin (CaN) activity, which suppresses Ca^{2+}-independent AKT-dependent eNOS signaling. Moreover, deletion of Ng in mice also reduced eNOS activity and caused endothelial dysfunction in flow-mediated dilation experiments. Our results demonstrate that Ng plays a crucial role in Ca^{2+}-CaM-dependent eNOS regulation and contributes to vascular remodeling, which is important for the pathophysiology of cardiovascular disease.

1. Introduction

Loss of vascular nitric oxide (NO) is a hallmark of endothelial dysfunction, promoting the pathophysiology of hypertension and atherosclerosis [1,2]. In the vasculature, NO is generated by endothelial nitric oxide synthase (eNOS) activity, which is highly regulated by calcium (Ca^{2+})-calmodulin (CaM) binding in the canonical CaM-binding domain, and aligns the oxygenase and reductase domains of eNOS [3]. In addition, phosphorylation of the regulatory Ser1177 site is required for eNOS activation. Ser1177 can be phosphorylated through a Ca^{2+}-dependent pathway by CaMKII or a Ca^{2+}-independent pathway by protein kinase A (PKA) and protein kinase B (AKT) [4,5].

The vascular AKT-mediated eNOS pathway responds directly to hemodynamic shear stress, a fractional force of blood flow on the endothelium, which leads to a series of intracellular biochemical signals that critically modulate endothelial cell function. Steady laminar flow is known to induce eNOS activation and NO production, maintain endothelial cell function, and decrease pro-inflammatory gene expression. In contrast, disturbed flow patterns, such as those observed at certain areas of the arterial tree; bifurcations and curvatures, result in a pro-inflammatory phenotype characterized by poor alignment, high turnover, endothelial activation, and enhanced pro-inflammatory gene expression [6,7]. Thus, comparing the two flow patterns is an effective way to elucidate the role of mechanotransduction related to Ca^{2+}-dependent and Ca^{2+}-independent eNOS pathways.

Neurogranin (Ng) is a known Ca^{2+} sensing molecule in the brain [8–10] and its genetic variant is significantly associated with several brain diseases [11–13]. Although it is believed that Ng is a brain-specific protein, our results, in accordance with evidence from the literature, suggest that Ng can be produced outside of the brain [14,15]. In this study, we are the first to identify significant Ng expression in vascular tissue, especially in endothelial cells. At high intracellular

Ca^{2+} concentrations, Ng demonstrates a strong affinity for CaM, with the result that Ng expression inhibits Ca^{2+}-CaM complex formation. This Ng-CaM binding affinity is regulated by PKCγ-dependent phosphorylation of the CaM binding site on Ng (S37) [16,17]. In addition, S-nitrosylation of Ng renders it a highly favorable acceptor of NO modification in four Cys residues, leading to dramatically attenuated CaM binding affinity and 2-fold weaker PKC substrate phosphorylation activity [8,10]. We have previously reported that a deficiency of Ng in the mouse brain increases CaMKII activity [18] and suppresses AKT activity [19], which causes a selective response to increased intracellular Ca^{2+}-CaM complex with a lack of Ng-CaM binding. These observations indicate that Ng could possibly play a role as a feedback regulator in Ca^{2+}-dependent NO signaling in endothelial cells.

Here, we report that Ng is uniquely expressed in the endothelial cells (EC) of human and mouse vasculature. Ng knockout mice demonstrate significantly decreased NO levels in the blood and present cardiac failure at the age of 10 months. Thus, Ng appears to be an important molecular component for eNOS regulation and cardiovascular function in the ECs. The goal of this study was to determine how Ng expression regulates Ca^{2+}-dependent eNOS signaling and vascular stability. To test the role of Ng in eNOS activation, a Ng knockdown in human aortic endothelial cells (HAEC) was performed using Ng SiRNA along with Ng knockout (Ng $^{-/-}$) in mice. Moreover, we examined the effect of Ng expression during both laminar and oscillatory using a shear stress model. Specifically, AKT-dependent eNOS phosphorylation and NF-κB-mediated eNOS expression were significantly regulated by Ng expression during the shear stress. These findings lead us to suggest a new target pathway for eNOS regulation that integrates the AKT pathway as a part of the Ca^{2+}-dependent eNOS and endothelial activation process.

2. Materials and methods

Cell culture, treatments, and tissues. Human aortic endothelial cells (HAEC; Lonza, San Diego, CA) were purchased and maintained in MCDB 131 medium supplemented with 10% FBS, 2 mmol/L glutamax, 10 U/mL penicillin (GIBCO/Life technologies, Carlsbad, CA), 100 μg/mL streptomycin (GIBCO/Life technologies, Carlsbad, CA), 30 μg/mL heparin sodium, and bovine brain extract (25 μg/mL). HAEC at 70% confluency were transfected with siRNA targeting Ng (50 nmol/L) using Lipofectamine 3000 (Life Technologies, Carlsbad, CA) for 8 h. Experiments were performed after 48 h. For the pharmacological experiments, HAECs cells were treated 1 h with the calcineurin inhibitors, Cyclosporin A 3 μM (Calbiochem, San Diego, CA), FK506 10 nM (Tocris Bioscience, Bristol, UK), NFAT inhibitor A-285222 10 μM (Abbott Laboratories (Abbott Park, IL), Phosphoinositide 3-kinases (PI3K) inhibitor LY-294002 1 μM (Sellechem, Houston, TX) and nitric oxide donor DETA NONOate 100 μM, (Tocris Bioscience, Bristol, UK). All experiments using Post-mortem human tissue were deemed nonhuman research by the local institutional review board.

Animals. Male Ng $^{-/-}$ mice (C57BL/6 J background, Jackson Laboratories, Bar Harbor, ME) aged 4-month-old and 10-month-old were used. Mice were group housed in standard Plexiglas cages under a 12 h light/dark cycle (lights on at 6:00 a.m.) at a constant temperature (24 ± 0.5 °C) and humidity (60 ± 2%) with food and water available *ad libitum*. The animal care and handling procedures were in accordance with LSUHSC institutional and National Institutes of Health (NIH) guidelines.

Shear stress assay. HAEC were transfected with either 50 nmol/L Ng siRNA or a mock control using Lipofectamine 3000 (Life Technologies, Carlsbad, CA). Briefly, cells (1×10^6) were plated on fibronectin (100 μg/ml) coated slides (Corning, Kennebunk, ME) to confluence and the slides placed in a flow chamber to be subjected to either laminar flow (10 dyn/cm^2) or oscillating flow as previously described [20]. In brief, oscillatory flow is generated using an infusion withdrawal pump (between +6 dyn/cm^2 and -4 dyn/cm^2; 1 Hz) with 2 dyn/cm^2 forward

flow superimposed by a peristaltic pump. After the cessation of flow, cells were lysed by addition of 2X Laemmli buffer (BioRad, Hercules, CA).

NO measurements. Nitric oxide (NO) metabolites (NOx) were measured using Sievers Nitric Oxide Analyzer 280i in Redox Molecular Signaling Core in LSUHSC-Shreveport. An aliquot of plasma was placed in NO preservation solution (800 mmol/L potassium ferricyanide, 17.6 mmol/L N-ethylmaleimide, 6% Nonidet P-40) for tri-iodide NO chemiluminescent analysis. Nitrite is reduced using the tri-iodide method. NO is measured using an ozone-based chemiluminescent assay (Sievers Nitric Oxide Analyzer 280i, Weddington, NC) [21]. Aliquots of samples were tested for sulfanilamide resistance following addition of an acidic sulfanilamide solution to a final concentration of 0.5% v/v and sitting in the dark for 15 min prior to injection into the analyzer. Brain tissue (positive control) was homogenized in 500 μL NO preservation buffer (1.25 mol/L potassium ferricyanide, 56.9 mmol/L N-ethylmaleimide, 6% Nonidet P-40 substitute in PBS).

Immunofluorescence. For immunofluorescence analysis, mouse tissues were fixed with formalin and embedded in paraffine. After xylene dewaxing and citrate buffer (10 mM, Vectors Biolabs, Malvern, PA) antigen retrieval, 5 μm sections were incubated with primary antibodies (1:200 dilution) overnight at 4 °C, followed by staining with Alexa Fluor 488-conjugated secondary antibody (4 μg/mL) (Life Technologies, Carlsbad, CA). Nuclei were counterstained with DAPI. Immunofluorescence images were acquired using LSM 710 Confocal microscope (Carl Zeiss, Oberkochen, Germany).

Western blot analysis. lysates from cells or mouse tissues were homogenized in a solution containing 50 mM Tris buffer (pH 7.4), 2 mM EDTA, 5 mM EGTA, 0.1% SDS, a protease inhibitor cocktail (Roche, Indianapolis, IN), and phosphatase inhibitor cocktail type I and II (Sigma, Saint Louis, MO). Homogenates were centrifuged at $500 \times g$ for 15 min and supernatants were collected. Proteins were analyzed using the Bradford protein assay (BioRad, Hercules, CA). Proteins were separated using 4–12% SDS-PAGE (Bio-Rad, Hercules, CA) at 130 V for 2 h, transferred onto PVDF membranes at 30 V for 1 h (BioRad, Hercules, CA), and incubated with antibodies against Neurogranin #07–425 and phospho Neurogranin #07–430 (Millipore, Billerica, MA), phospho ENOS #PA5-17917 and phospho NFAT #PA5-64484 (Invitrogen, Carlsbad, CA, USA), phospho AKT #9271, AKT #9272, ENOS #3202, NFAT-1 #5861, phospho NFκB #3033, NFκB #8242, phospho ERK #9106, and GAPDH #SC-32233 (Santa Cruz, Dallas, TX). Chemiluminescent bands were detected on an Image Station and quantified using NIH Image J software.

Cardiac imaging and flow-mediated vasodilation. Using a Visual Sonics Vevo 3100 imaging system, two-dimensional and motion-mode (M-mode) transthoracic echocardiography was performed in both Ng $^{+/+}$ mice and Ng $^{-/-}$ mice (10-month-old). Mice were under anesthesia with isoflurane gas and physiological response of the heart was assessed. Off-line analyses were conducted to measure in diastole of interventricular septum (IVSd) and left ventricular free wall (LVFWd) thickness and the left ventricular (LV) end diastolic and end systolic internal dimensions (LVIDd and LVIDs) [22]. Using high-frequency ultrasound, we characterized endothelium-dependent dilation of the femoral artery after temporal ischemia of the lower part of the hindlimb of Ng $^{+/+}$ mice and Ng $^{-/-}$ mice (4-month-old) [23]. The ultrasound probe was attached to a stereotactic holder and was manually aligned with the femoral vein visible at the upper inner thigh. A vascular occluder (5 mm diameter, Harvard Apparatus) was placed around the proximal hindlimb to induce occlusion of the distal hindlimb as an ischemic trigger. Following hindlimb ischemia for 1-min, the cuff was deflated and femoral artery diameter measurements were continuously recorded for 5 min at 30 s intervals.

Statistical analysis. All data are expressed as mean ± standard error of the mean (SEM). Statistics were performed using either a two-tailed Student's *t*-test (Prism, GraphPad Software, La Jolla, CA) or two-way repeated measures ANOVA followed by a Tukey *post hoc* test
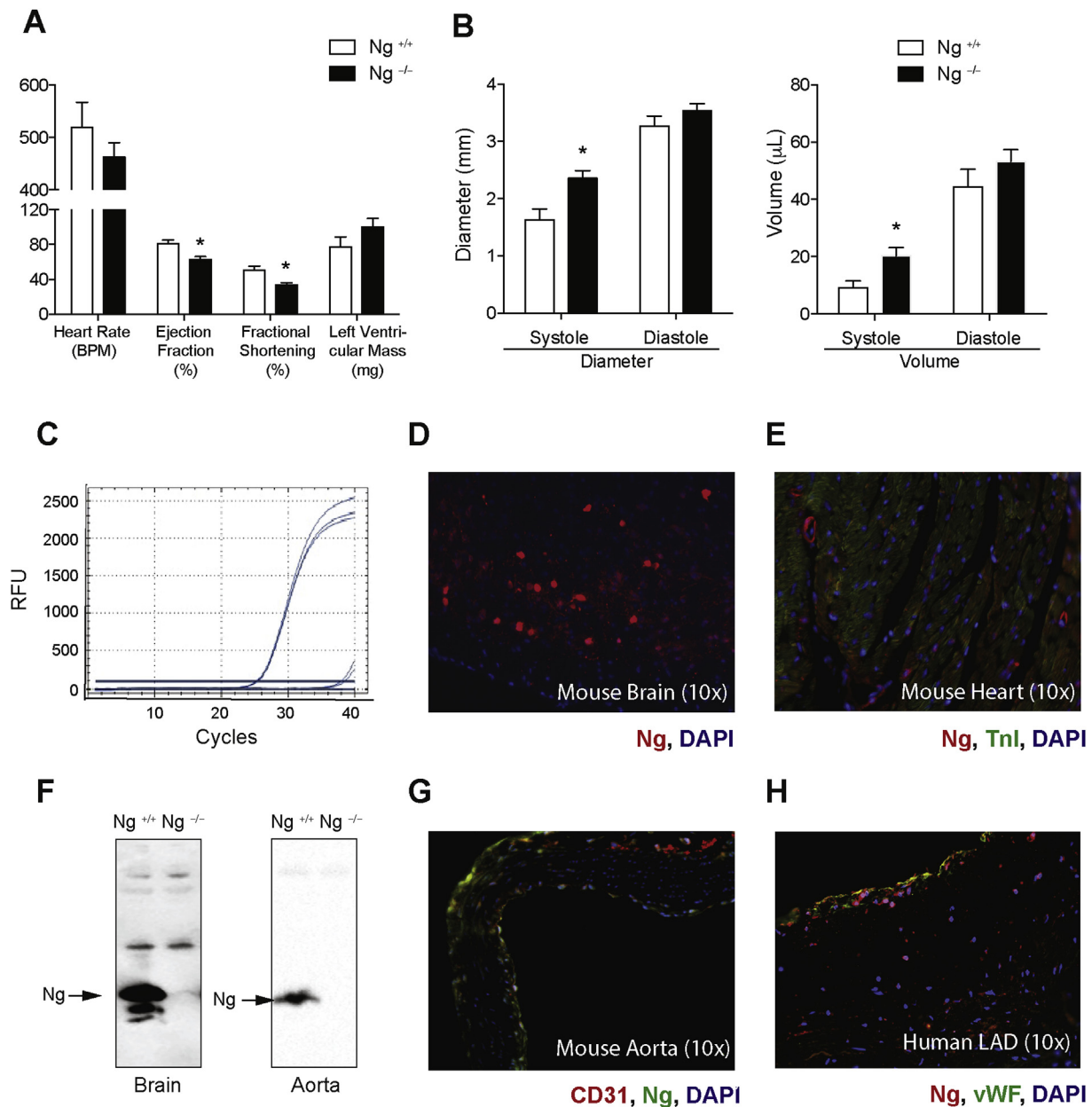
Fig. 1. Ng expressed in the endothelium and Ng $^{-/-}$ mice demonstrate heart failure. (A) 10-month-old Ng $^{-/-}$ mice demonstrated decreased ejection fraction and fraction shortening compared to Ng $^{+/+}$ mice. (B) Both systolic diameter and volume were significantly increased in Ng $^{-/-}$ mice. (C) Ng mRNA expression in the heart of Ng $^{+/+}$ mice assessed using RT-PCR. (D) Ng protein is significantly expressed in the brain of Ng $^{+/+}$ mice. (Ng: red; DAPI: blue) (E) Ng protein is significantly expressed in the perivascular areas of the heart of Ng $^{+/+}$ mice with little co-staining with a cardiomyocyte marker, troponin I (Ng: red; DAPI: blue; TnI: green). (F) Ng protein expression in the brain and aorta assessed using Western blotting. (G) Ng expression in the aorta is co-localized with an endothelial marker, CD31 (Ng: green; DAPI: blue; CD31: red). (H) Ng is significantly expressed in the LAD of human postmortem tissues. Ng protein is co-localized with an endothelial marker, von Willebrand factor (Ng: red; DAPI: blue; vWF: green). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(SigmaStat, SYSTAT software, Point Richmond, CA). The criterion for statistical significance was $p < 0.05$.

3. Results

3.1. Neurogranin is expressed in the vascular system in both human and mice

We observed that 10-month-old Ng $^{-/-}$ mice demonstrated heart failure with significantly less ejection fraction and fraction shortening (Fig. 1A), with enlarged heart diameter and volume (Fig. 1B). Although Ng is known as brain specific protein, we measured Ng expression in the heart to determine the role played by Ng in the pathophysiology of cardiovascular diseases. We found significant Ng mRNA expression in wild-type mouse heart tissue using RT-PCR (Fig. 1C). However, we could not demonstrate Ng protein expression in wild-type heart tissue using Western blotting. Therefore, we conducted immunofluorescence (IFF) studies to validate Ng protein expression in the wild-type mouse heart. IFF results indicated that Ng protein is highly expressed in brain neurons (Fig. 1D) and in the perivascular area in wild-type mouse heart tissue (Fig. 1E). Since Ng expression was not observed in cardiomyocytes, we further investigated Ng expression in the vasculature, focusing on the aorta. We found Ng significantly expressed in the aorta of wild-type mice using Western blotting (Fig. 1F). We then validated
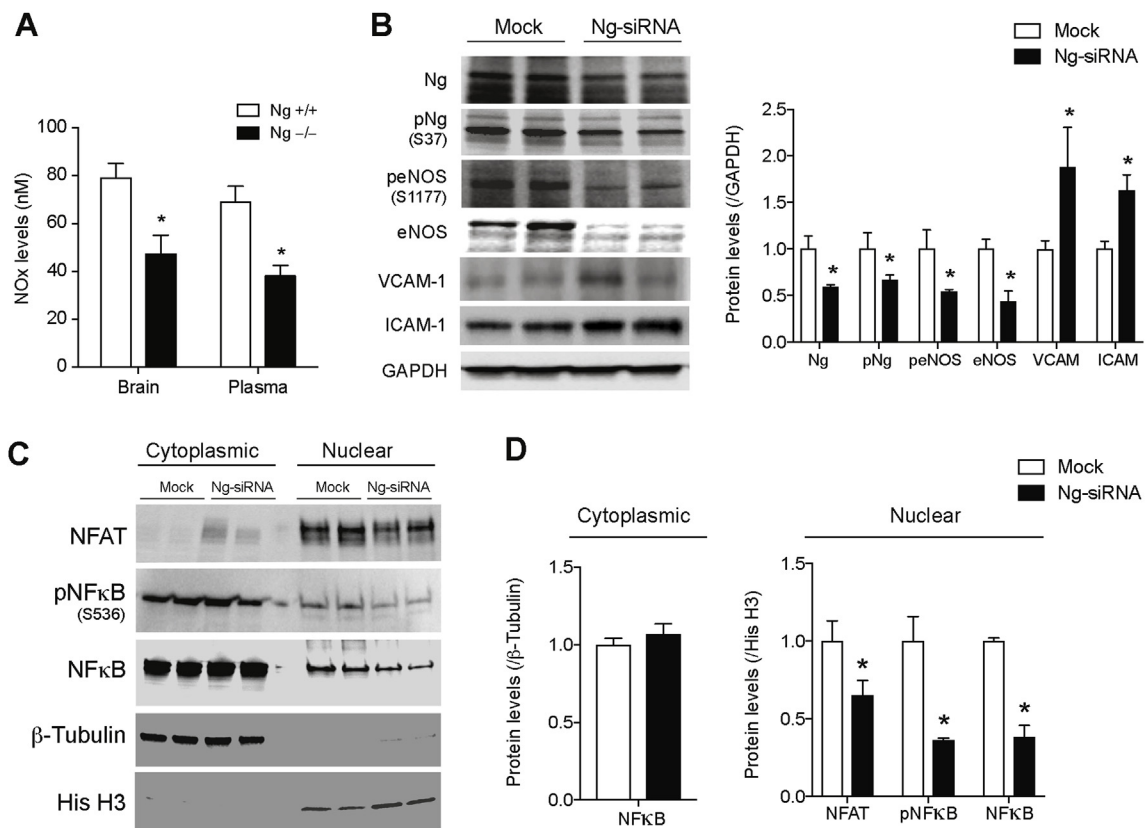
Fig. 2. Lack of Ng decreases eNOS signaling in both *in vitro* and *in vivo*.
(A) Ng $^{-/-}$ mice showed significantly decreased NOx levels in the brain and plasma (n = 4). (B) Ng siRNA in HAEC decreases Ng expression, Ng phosphorylation, eNOS phosphorylation (S1177), and eNOS expression, while it increases adhesion molecular expression such as V-CAM1 and I-CAM1 (n = 4). (C and D) Ng siRNA decreases both NFAT and NF-κB nuclear localization, which represents transcriptional activity (n = 4).

endothelial Ng expression in the mouse aorta (Fig. 1G) and left anterior descending artery (LAD) in human postmortem tissues (Fig. 1H). This is the first evidence that confirms Ng expression in endothelial cells. Since Ng in the brain plays an essential role in Ca^{2+}-CaM signaling and NO production, we hypothesized that Ng may play an important role in Ca^{2+}-dependent eNOS signaling and endothelial activation, which is critical for the development of cardiovascular disease.

3.2. Lack of Ng decreased NO levels through decreased eNOS activation

Having shown that Ng $^{-/-}$ mice have a detrimental effect on cardiac function, we postulated if Ng in the endothelium alters NO levels. Using a nitric oxide analyzer, we assessed NO levels in Ng $^{-/-}$ mice. We found significantly reduced NO levels in both the plasma and the brain of Ng $^{-/-}$ mice compared to those in wild type (Ng $^{+/+}$) animals (Fig. 2A). To understand how a lack of Ng decreases NO regulation in the endothelial cell, we generated an *in vitro* Ng knockdown model using Ng siRNA in human aortic endothelial cells (HAEC). Transfection efficiency was assessed using Western blotting and showed significantly less Ng expression and Ng phosphorylation compared to mock controls (Fig. 2B). Then, we assessed whether a lack of Ng decreases eNOS signaling by measuring eNOS phosphorylation (S1177) and eNOS expression. Interestingly, both eNOS phosphorylation and eNOS expression is significantly decreased by Ng siRNA, which explains the reduction of NO levels in the plasma of Ng $^{-/-}$ mice. Resting endothelium suppress the expression of adhesion molecules such as VCAM-1 and ICAM-1 and that is to some extent is played by NO [24]. Therefore, we examined whether Ng knocking down induces endothelial activation by measuring endothelial adhesion molecular expression change. Both intercellular endothelial adhesion molecule 1

(ICAM-1) and vascular endothelial adhesion molecule 1 (VCAM-1) were significantly upregulated, suggesting that a lack of Ng decreased NO levels in HAEC and enhanced endothelial activation (Fig. 2B).

Because a lack of Ng decreases NO generation, as well as increases ICAM-1 and V-CAM1, we examined the activation of both NF-κB and NFAT, which regulates eNOS expression and the pro-inflammatory response in endothelial cells [25,26]. While we did not observe significant changes in the cytosolic levels, we found in the nucleus that both NFAT and NF-κB phosphorylation (S536) were significantly decreased by Ng siRNA treatment (Fig. 2C and D). Importantly, this finding is positively associated with decreased eNOS expression (Fig. 2B), which is one product of NF-κB or NFAT mediated gene expression [27]. Although both ICAM-1 and VCAM-1 are known to be activated by the NF-κB mediated pro-inflammatory response [26], our finding suggests that a lack of Ng dramatically decreased NO regulation in the HAEC, promoting endothelial activation by a mechanism other than NFAT or NF-κB signaling.

3.3. Neurogranin regulates endothelial activation in response to short-term shear stress

Since flow patterns induce endothelial activation and NO dependent vasodilation [28], we examined how Ng expression regulates eNOS function and expression during shear stress. HAEC were exposed to short-term laminar flow to assess early endothelial activation and Ng expression was assessed using Western blotting. Ng protein expression was significantly decreased by short-term shear stress in the mock controls and Ng siRNA also demonstrated a further reduction in Ng expression (Fig. 3A and B).

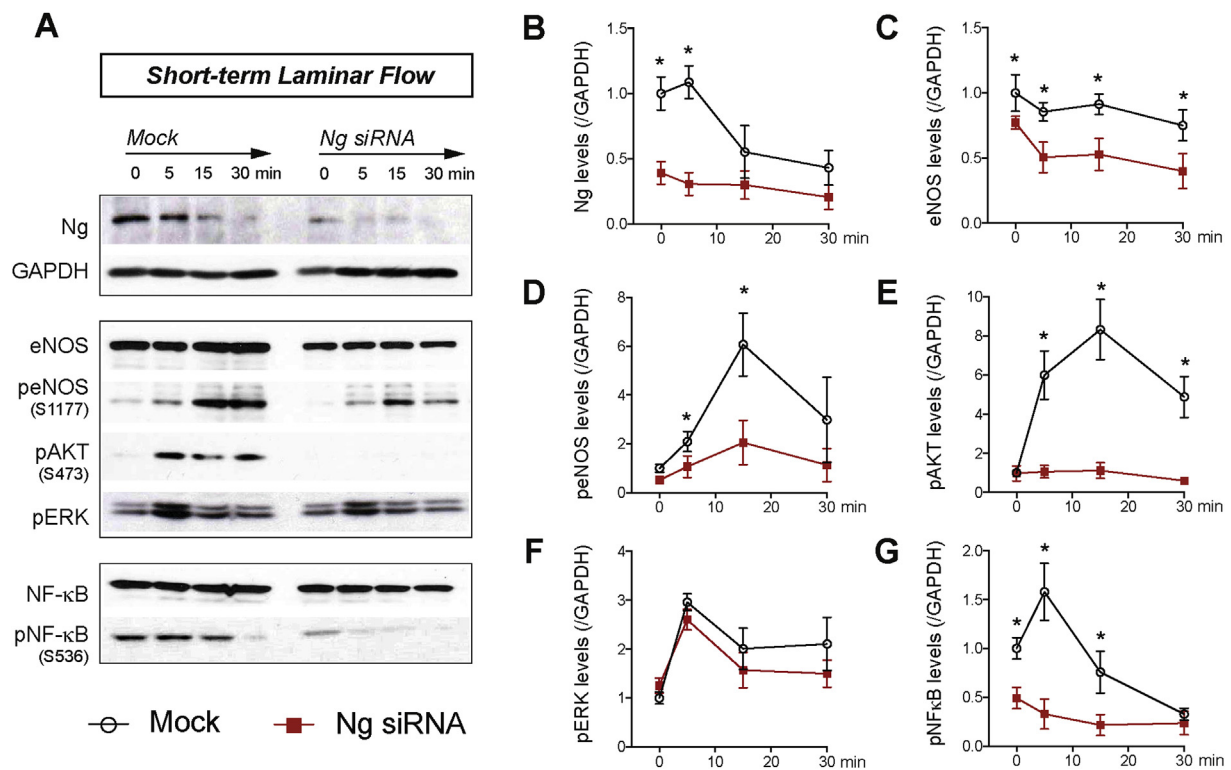We then examined whether a lack of Ng coupled with shear stress

Fig. 3. Shear stress regulates Ng expression and suppresses ATK-eNOS phosphorylation in Ng siRNA. (A) HAEC were exposed to short-term laminar flow for 30 min (n = 4). (B) Ng expression is significantly decreased by laminar flow in both mock and Ng siRNA. (C) eNOS expression was significantly decreased by Ng siRNA but eNOS expression in Ng siRNA was not increased by shear stress. (D) Ng siRNA decreases eNOS phosphorylation (S1177) during shear stress. (E) AKT phosphorylation (S473) was suppressed by Ng siRNA, while shear stress activated AKT phosphorylation in a mock sample. (F) pERK did not show any changes between mock and Ng siRNA. (H) NF-κB phosphorylation was suppressed by Ng siRNA, while NF-κB expression did not show any changes. Data presented as mean ± standard error mean. *$p < 0.05$ by unpaired t-test compared to mock control.

affects eNOS expression and eNOS phosphorylation (Ser 1177). Both eNOS expression levels and eNOS phosphorylation was significantly lower in Ng siRNA (Fig. 3A) and in response to short-term laminar flow (Fig. 3C and D). Because AKT is a known upstream kinase for eNOS phosphorylation [29], AKT expression and AKT phosphorylation (S473) were assessed after exposure to short-term laminar flow. Interestingly, while Ng siRNA significantly suppresses activation of AKT phosphorylation (Fig. 3A and E), there was no difference in AKT expression. In addition, there was no change in extracellular signal regulated kinase 1/2 (ERK1/2) signaling (Fig. 3A and F). This finding supports the hypothesis that a lack of Ng dominantly suppresses AKT activation and subsequent eNOS activation and plays an essential role in mechanotransduction.

Finally, we examined NF-κB signaling, which potentially regulates eNOS expression and the pro-inflammatory response during short-term laminar flow. There was no difference in NF-κB expression between mock and Ng siRNA; however, Ng siRNA in endothelial cells demonstrated the suppression of NF-κB activation, as assessed by phosphorylation of the S536 in NF-κB during short-term laminar flow (Fig. 3A and G). There was no significant change in NFAT signaling during short-term shear stress. Overall, a lack of Ng suppresses NF-κB mediated eNOS signaling, which possibly induces endothelial activation during shear stress.

3.4. Neurogranin regulates endothelial activation by long-term shear stress

Sustained endothelial activation was observed in response to long term oscillatory shear stress [20] therefore, we examined the eNOS signaling change after long-term (18 h) oscillating flow compared to long-term (18 h) laminar flow (Fig. 4A). This comparison enabled us to determine whether Ng expression played an atheroprone or

atheroprotective role. Following laminar flow exposure, Ng expression did not change, however, Ng levels significantly decreased during exposure to oscillating flow (Fig. 4B). Two-way ANOVA identified a significant effect of Ng siRNA ($F(1,8) = 10.72, p < 0.01$) and interaction between flow and Ng siRNA ($F(1,8) = 12.67, p < 0.01$). We also determined how a lack of Ng alters eNOS phosphorylation, eNOS expression, and endothelial activation during the oscillating flow.

AKT phosphorylation was significantly decreased in Ng siRNA as well as during oscillating flow. Two-way ANOVA identified a significant effect of Ng siRNA ($F(1,8) = 7.48, p < 0.05$) and flow types ($F(1,8) = 6.61, p < 0.05$) (Fig. 4C), although there was no change in AKT protein expression (Fig. 4D). eNOS phosphorylation was significantly decreased during oscillating flow compared to laminar flow. Two-way ANOVA identified a significant effect of Ng siRNA ($F(1,8) = 12.67, p < 0.01$) and an interaction between flow and Ng siRNA ($F(1,8) = 10.72, p < 0.05$) (Fig. 4E). Although eNOS expression was significantly decreased by Ng siRNA, oscillating flow increased eNOS expression. Two-way ANOVA identified a significant interaction between flow and Ng siRNA ($F(1,8) = 12.49, p < 0.01$) (Fig. 4F). Consistent with the change in eNOS expression, NF-κB phosphorylation also demonstrated a similar pattern with a statistical interaction between flow and Ng siRNA ($F(1,4) = 11.93, p < 0.05$), which may provide evidence that eNOS expression is regulated by NF-κB activation (Fig. 4G).

Finally, we examined both ICAM-1 and VCAM-1 expression in response to long-term oscillating flow and Ng siRNA. Two-way ANOVA analysis demonstrated that ICAM-1 expression was significantly increased by the oscillating flow ($F(1,10) = 10.81, p < 0.01$) and Ng siRNA ($F(1,10) = 5.27, p < 0.05$) (Fig. 4H). Similarly, VCAM-1 expression was significantly increased by oscillating flow ($F(1,8) = 8.52, p < 0.01$) and Ng siRNA ($F(1,8) = 5.95, p < 0.05$) (Fig. 4I). Overall,
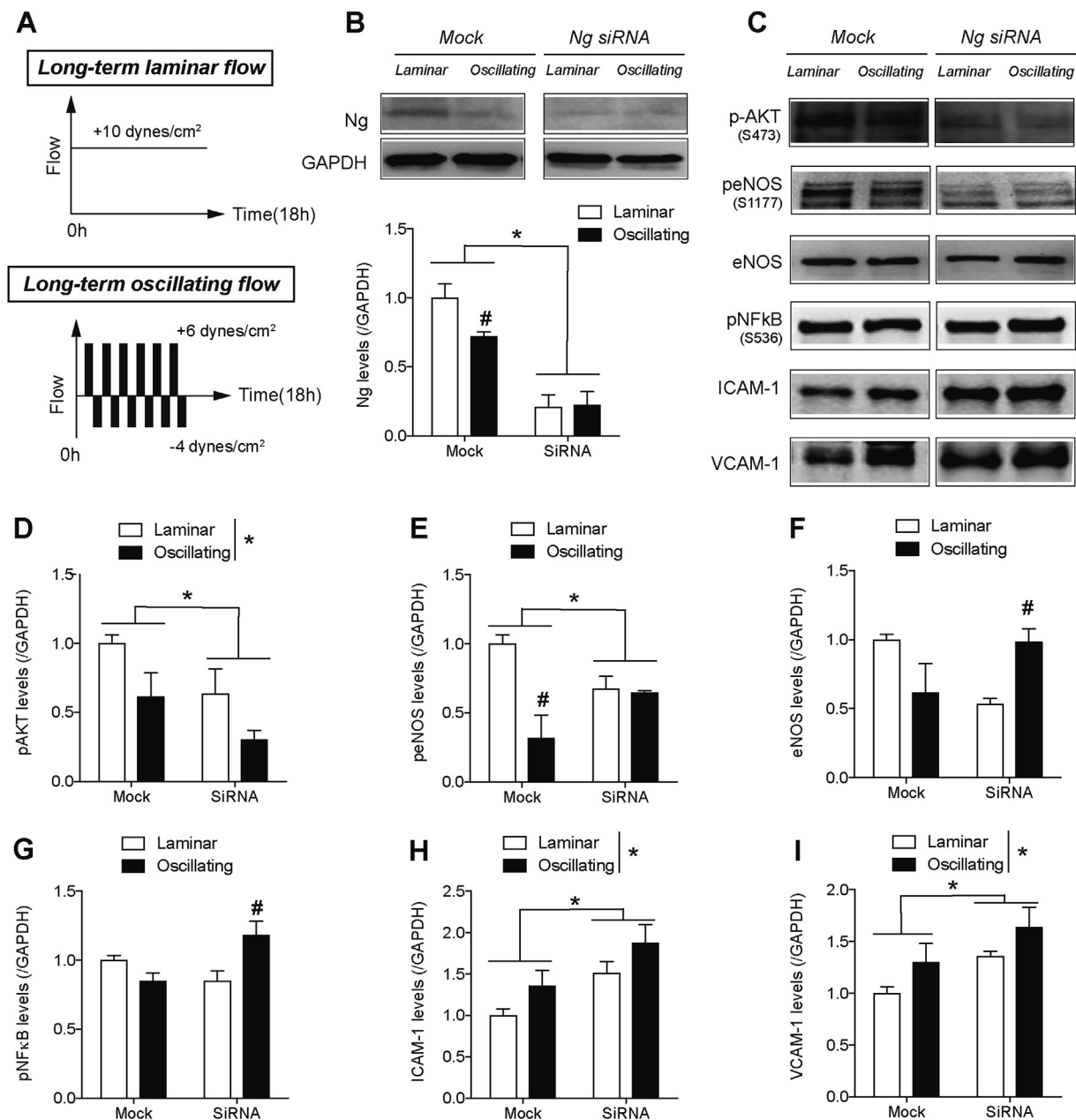
Fig. 4. Long-term oscillating flow decreases Ng expression, suppresses eNOS regulation, and activates adhesion protein. (A) Schematics of long-term laminar flow (18 h) and long-term oscillating flow (18 h) experiments. (B) Long-term oscillating flow significantly decreased Ng expression compared to long-term laminar flow. (C) Both mock and Ng siRNA HAEC were exposed to long-term laminar flow and long-term oscillating flow (n = 3–4). (D) AKT phosphorylation was decreased by both siRNA and oscillating flow. (E) eNOS phosphorylation was decreased by siRNA. Oscillating flow in the mock sample decreased eNOS phosphorylation. (F) eNOS expression is increased by oscillating flow in Ng siRNA. (G) NF-κB phosphorylation is increased by oscillating flow in Ng siRNA. (H and I) both ICAM-1 and VCAM-1 are increased by siRNA and oscillating flow. Data presented as mean ± standard error mean. *$p < 0.05$ by 2-way ANOVA compared to oscillating flow and Ng siRNA. #$p < 0.05$ by 2-way ANOVA compared to interaction between oscillating flow and Ng siRNA.

the combination of Ng siRNA and long-term oscillation flow promotes endothelial activation through AKT-eNOS signaling.

3.5. Lack of Ng decreased AKT activity and increased endothelial activation in the mouse aorta

Throughout our shear stress system, we observed that a lack of Ng suppresses AKT phosphorylation, which is mediated by Ca^{2+}-dependent calcineurin (CaN) regulation [30,31]. Therefore, we tested the Ng-mediated AKT activity after pharmacological treatment (Fig. 5A). First, we measured whether a lack of Ng alters CaN-mediated NFAT regulation. We observed that CaN inhibitors (Cyclosporine A, 3 μM, 1 h and

FK506l, 10 nM, 1 h)) decrease nuclear NFAT expression in both mock and Ng siRNA samples [27], while Ng siRNA decreases NFAT nuclear localization (Fig. 2D). We also tested how a lack of Ng diminishes AKT phosphorylation (S473) in HAEC after treatment with an NFAT inhibitor, CaN inhibitors, a PI3K inhibitor, and an NO donor. We demonstrated that AKT phosphorylation (S473) was significantly decreased by a PI3K inhibitor (LY294002, 1 μM, 1 h) in both mock and Ng siRNA. Interestingly, CaN inhibitors (Cyclosporine A and FK606) selectively decreased AKT phosphorylation (S473) in Ng siRNA samples, which implies that Ng expression modulates CaN-dependent AKT activity in endothelial cells.

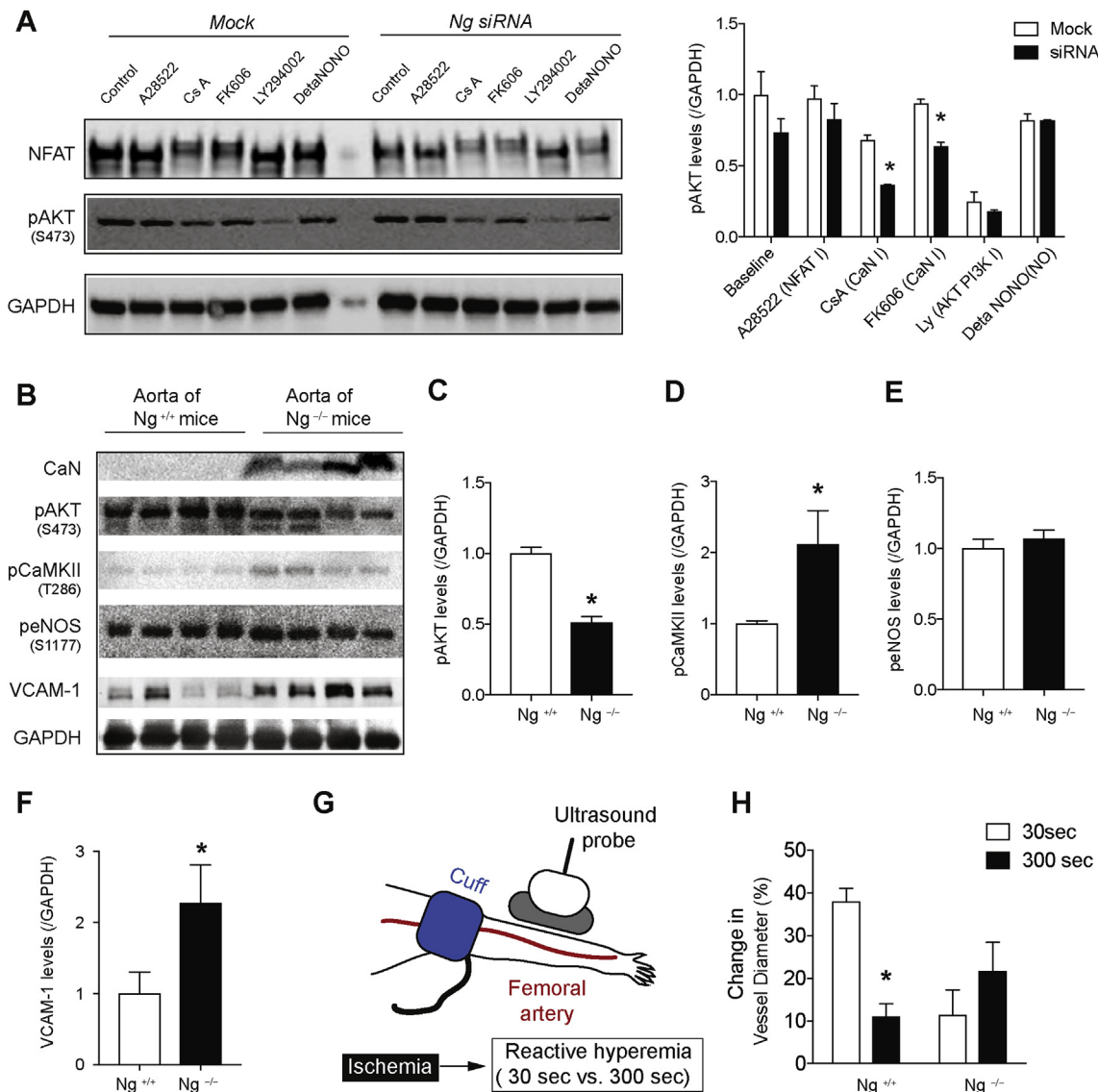Next, we tested how a lack of Ng regulates the CaN-mediated AKT

Fig. 5. Lack of Ng suppresses Ca²⁺-dependent AKT-eNOS regulation and endothelial dysfunction in mice. (A) The effect of pharmacological regulation in the Ca^{2+}-dependent eNOS pathway (A28522: NFAT inhibitor, Cyclosporine A and FK606: CaN inhibitor, LY294002: PI3K inhibitor, DetaNONOate: NO donor). NFAT nuclear localization was decreased by CaN inhibitors (Cs A and FK606) in both mock and Ng siRNA. AKT phosphorylation was decreased by a PI3K inhibitor (LY294002) in both mock and Ng siRNA. CaN inhibitors (Cs A and FK606) significantly decreased AKT phosphorylation in Ng SiNRA compared to mock SiRNA, which implies that a lack of Ng modulates the CaN-AKT pathway (n = 3). (B) Lack of Ng activated Ca^{2+}-dependent CaN expression in the aorta of Ng $^{-/-}$ mice (n = 4). (C) Lack of Ng in mice significantly decreased AKT phosphorylation (S473) in the aorta. (D) Ca^{2+}-dependent CaMKII phosphorylation (T286) was increased in the aorta of Ng $^{-/-}$ mice. (E) There was no change in the eNOS phosphorylation between Ng $^{+/+}$ mice and Ng $^{-/-}$ mice. (F) Ng $^{-/-}$ mice demonstrated increased adhesion molecule (VCAM-1) in the aorta. (G) Flow-mediated dilation (FMD) was performed in the femoral artery of Ng $^{+/+}$ mice and Ng $^{-/-}$ mice. (H) Ng $^{+/+}$ mice demonstrated significant femoral artery diameter changes between 30 s and 300 s after ischemia, while there was no difference in the Ng $^{-/-}$ mice (n = 4). Data presented as mean ± standard error mean. *$p < 0.05$ by paired t-test.

pathway in the mouse aorta, which may represent the physiological response of endothelial cells under chronic blood flow. Aortae were isolated from both Ng $^{+/+}$ mice and Ng $^{-/-}$ mice and the change in protein expression was measured using Western blotting (Fig. 5A). Since a lack of Ng is responsible for more Ca^{2+}-CaM complex formation, we observed significantly increased Ca^{2+}-dependent phosphatase calcineurin (CaN) expression (Fig. 5A and C), which significantly decreases AKT phosphorylation (S473) (Fig. 5C). In addition, Ng depletion increases Ca^{2+}-dependent kinase CaMKII phosphorylation (T287) (Fig. 5D). Since both CaMKII and AKT are known to regulate eNOS phosphorylation (S1177) in endothelial cells [3], we measured eNOS phosphorylation (S1177) in the aorta and found no difference between Ng$^{+/+}$ mice and Ng$^{-/-}$ mice (Fig. 5E). AKT and CaMKII may compromise eNOS phosphorylation (S1177) in the aorta of Ng $^{-/-}$ mice,

although we observed decreased NO levels in the Ng$^{-/-}$ mice.

3.6. Lack of Ng induces endothelial dysregulation in flow-mediated dilation

To further investigate the role of Ng signaling in endothelial activation, we assessed VCAM-1 expression in the aorta of Ng $^{-/-}$ mice. We found that the lack of Ng significantly increased VCAM-1 expression, which could explain the reduced NO levels in Ng mull mice (Fig. 5B and F). Overall, our finding provides with evidence that a lack of Ng controls eNOS signaling through the CaN-dependent AKT pathway rather than the CaMKII pathway.

Since our molecular results from both *in vitro* and *in vivo* experiments demonstrated that a lack of Ng dysregulates eNOS function, we tested whether Ng deletion impairs endothelial function *in vivo* using

flow-mediated dilation (FMD) model in Ng $^{-/-}$ mice, using high-frequency ultrasound [23]. Endothelium-dependent dilatation of femoral artery is almost exclusively dependent on stimulation of eNOS in mice [23]. Therefore, we measured femoral artery diameter and blood flow velocity at baseline and over 5 min during reactive hyperemia after an ischemia induced by an inflatable cuff near the lower limb. Interestingly, Ng $^{-/-}$ mice demonstrated a delay in the FMD response compared to that of Ng $^{+/+}$ mice. Two-way ANOVA showed that there is significant interaction between genotype and vessel diameter change (F $(1,6) = 9.66$, $p < 0.05$) (Fig. 5G). Ng $^{-/-}$ mice demonstrated an insufficient dilation change between 30 s and 300 s after ischemia due to defective endothelial function, while the vessel diameters of Ng $^{+/+}$ mice were restored to baseline (Fig. 5H).

4. Discussion

In this study, we first identified Ng expression in both human and mouse endothelium and showed how it regulates endothelial function. Interestingly, our preliminary data demonstrated that Ng $^{-/-}$ mice with decreased peripheral NO levels show endothelial dysfunction, which suggests an as yet unknown role for Ng in endothelial activation and cardiovascular disease. Moreover, Ng, a known Ca^{2+}-CaM modulator, specifically regulates CaN-dependent AKT signaling, which is significant with respect to eNOS biology and the pathophysiology of endothelial activation and dysregulation.

Although it is believed that Ng is a brain-specific protein, our results are in agreement with evidence in the literature and suggest that Ng can be produced outside of the brain [14,15]. Previous large-scale transcript analysis identified Ng expression in human glomerular microvascular endothelial cells (HGMEC) [32] and murine T-cells [14] and verified its expression using real-time polymerase chain reaction (RT-PCR) and immunohistochemistry. A recent study showed that Ng is also expressed in mammalian skeletal muscle and inhibits calcineurin by sequestering CaM [15]. Clinically, Ng is associated with a number of neurological diseases, including schizophrenia and Alzheimer's disease. Human genomics studies have reported that Ng genetic variants (rs12807809) or decreased Ng mRNA expression correlate with an increased risk of schizophrenia [12,33–36]. Ng $^{-/-}$ mice consistently demonstrate decreased spatial learning and memory [17] and decreased sensorimotor gating [37] mediated by glutamate-mediated Ca^{2+}-CaM signaling [18,38] and AKT signaling [19]. Thus, this study suggests that an increased risk for cardiovascular disease in individuals with neurological disease warrants further investigation [39,40]. This co-morbidity could be due to lifestyle factors, cardiovascular disease risk factors, or direct adverse cardiovascular effects caused by antipsychotic medications. Some antipsychotic agents, such as clozapine, have marked effects on the autonomic nervous system, leading to decreased heart rate variability and an increased risk of cardiac arrhythmias [41]. However, average compliance with antipsychotic drug regimens in schizophrenia is barely 50%, indicating that Ng induced cardiotoxicity, as a genetic risk factor, could possibly explain higher cardiac failure observed in patients with schizophrenia. Therefore, we postulate that Ng regulates both brain and cardiovascular function through a Ca^{2+}-CaM dependent pathway.

Our new finding, that Ng expression in the endothelium, is critical for the cardiovascular function since Ca^{2+}-CaM regulation plays an essential role in eNOS activation. Moreover, Ng contains redox active Cys residues that are oxidized by NO [8,10]. Due to activation of Ca^{2+}-dependent eNOS, the subsequent NO modification of Ng favors its dissociation from the Ng-CaM complex, which means that Ng can be considered a Ca^{2+} sensitive NO signaling regulator. We reported that Ng expression is decreased by shear stress and Ng knockdown dampens eNOS activity in experiments using in vitro HAEC. This Ng mechanical study provides us with a new understanding of mechanotransduction and its significant potential to be involved in the pathophysiology of vascular remodeling. Furthermore, a lack of Ng significantly suppresses

AKT phosphorylation activated by shear stress; as a result, we believe that AKT is part of Ca^{2+}-dependent eNOS regulation. As it applies to shear stress in general, PI3K/AKT-mediated eNOS regulation has been reported in several rodent models, but our finding suggests the importance of the Ng-AKT pathway [19] as a possible new eNOS mechanism located distal from the Ng-CaN pathway [15]. For example, endothelial specific AKT knockout mice demonstrate significantly impaired blood flow recovery [42], which was also observed in Ng $^{-/-}$ mice. Consistently, AKT phosphorylation is significantly decreased in the aorta of Ng $^{-/-}$ mice; our findings support the importance of suppression of AKT phosphorylation in endothelial regulation and cardiovascular disease. Our results suggest that AKT-eNOS regulation, which is known as a Ca^{2+}-independent eNOS pathway may instead involve a Ca^{2+}-dependent pathway through Ng-CaN signaling.

NF-κB and NFAT signaling regulates eNOS expression and the pro-inflammatory response during shear stress. The lack of Ng also decreases both NF-κB and NFAT signaling in HAEC, which may contribute to decreased eNOS expression. In particular, NF-κB phosphorylation was significantly decreased with Ng siRNA treatment during short-term laminar flow, implying that a lack of Ng decreases the IKK beta dependent canonical pathway. On the other hand, after long-term oscillation flow, Ng siRNA increases NF-κB phosphorylation. These changes in NF-κB phosphorylation were consistent with changes in eNOS expression, while there was no significant difference in NFAT signaling during shear stress. Thus, how the lack of Ng expression or subsequent Ca^{2+}-CaM dysregulation can regulate the NF-κB canonical pathway requires further investigation. Overall, Ng expression in HAEC controls CaN-AKT signaling and NF-κB activation, which is important for the endothelial activation process.

Since the shear stress decreased Ng expression in HAECs, we examined how Ng-mediated eNOS signaling responds to flow-mediated dilation (FMD), and compared these results with those from the aortas of Ng $^{-/-}$ mice. There were some discrepancies between in vitro (HAEC) and in vivo (mouse aorta) results; however, we demonstrated that a lack of Ng decreases AKT-mediated NO production and increases adhesion molecular (VCAM-1 and ICAM-1) expression, supporting endothelial activation. Moreover, our FMD results demonstrated that Ng $^{-/-}$ mice displayed defective endothelial function with a delay in dilation response during reactive hyperemia. Although endothelial cells (EC) and vascular smooth muscle cells (VSMC) are primarily the two major cell types in blood vessels, Ng expression in the endothelial cells plays an essential role in sustaining vascular homeostasis. Therefore, an endothelial-specific Ng knockout mouse strategy is required to validate our molecular finding and to identify the pathophysiology of Ng-mediated cardiovascular disease.

Although FMD stimulated by reactive hyperemia allows us to identify Ng-mediated endothelial dysregulation in Ng $^{-/-}$ mice, Ng-mediated endothelial function needs to be further validated [43]. For example, measuring blood arterial pressure change using telemetry will help to determine endothelial function in response to endothelial vasodilators. The comparison of pharmacological treatment between acetylcholine (endothelial-dependent) and sodium nitroprusside (endothelial-independent) will be beneficial to elucidated the role of Ng in NO-mediated endothelial function [44]. This approach also enables us to infer whether Ng expression is affected by NO-mediated blood pressure changes or not. In addtion, arterial stiffness assessment will be a possible approach to measuring Ng-mediated endothelial dysregulation. Since eNOS inhibition increases aortic stiffness [45], rodent pulse wave velocity (PWV), which measures travel time of the pulse between aortic arch and abdominal aorta, will help us to determine whether Ng expression is required to maintain normal vascular compliance.

In the endothelium, we expect that Ng acts as a scaffolding protein that governs the dynamics of eNOS, since Ng is known to sequester CaM and CaN in the brain [46,47]. The colocalization study between Ng and eNOS in the transmembrane will enable us to determine how Ng expression regulates Ca^{2+}-dependent eNOS activity and expression. In

addition, the role of PKC must be further examined since PKC is known to inhibit both Ng function by phosphorylation (S37), and eNOS function by phosphorylating eNOS (T495). PKC activation may be a possible supporting mechanism suggesting how the depletion of Ng suppresses eNOS function, since the activation of vascular PKC inhibits AKT-dependent eNOS function and promotes endothelial activation [48]. In addition, it needs to be further investigated how endothelial Ng signaling fits with other known eNOS modulators such as chemicals (acetylcholine) and hormones (vascular endothelial growth factor; VEGF) [49]. Acetylcholine receptor activation in the endothelium is known to increase the levels of intracellular Ca^{2+} concentration so that acetylcholine may activate PKC-mediated Ng phosphorylation and CaMKII-dependent eNOS phosphorylation. Or, Ng expression in the endothelium may be critical for VEGF-mediated eNOS regulation, since VEGF activates the PI3K-AKT pathway. These pathways are relevant to our finding that depletion of Ng suppresses AKT activation during shear stress. Therefore, it needs to be highlighted that the Ng-eNOS pathway will be a novel therapeutic target for endothelial activation by balancing chemicals, hormones, and shear stress.

Endothelial activation is known to promote vasoconstriction, smooth muscle cell proliferation, leukocyte adhesion, and the LDL oxidation process, all of which lead ultimately to cardiovascular disease. So far, it is not known whether Ng dysregulation promotes atherosclerosis, coronary artery disease, or hypertension. Nevertheless, our study is the first to demonstrate that Ng expression is critical for endothelial AKT activation and subsequent eNOS activation, which are critical to maintaining vascular stability and remodeling. In addition, AKT expression in the endothelium regulates vascular stability and function through vascular smooth muscle apoptosis [50]. Therefore, using an endothelial specific Ng knockout mouse enabled us to elucidate whether decreased AKT activity in the aorta is controlled by PI3K/mTOR phosphorylation or CaN mediated dephosphorylation [51].

In conclusion, Ng plays an important role in eNOS regulation and endothelial activation. We first identified Ng expression in both human and mouse endothelial cells. Ng expression is decreased by shear stress and lack of Ng significantly suppresses AKT-mediated eNOS regulation. These findings suggest that Ng promotes Ca^{2+}-dependent phosphatase activity that suppress AKT-dependent eNOS regulation and NO production in the endothall cell. Ng knockout mice exhibit endothelial dysfunction in FMD as well as endothelial activation in the aorta. Thus, it is required to further study the role of Ng-NO pathway and cardiovascular disease.

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Disclosures

All authors declare no conflicts of interest.

Declaration of competing interest

All authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101487.

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