The F-BAR Protein NOSTRIN Dictates the Localization of the Muscarinic M3 Receptor and Regulates Cardiovascular Function

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Rationale: Endothelial dysfunction is an early event in cardiovascular disease and characterized by reduced production of nitric oxide (NO). The F-BAR protein NO synthase traffic inducer (NOSTRIN) is an interaction partner of endothelial NO synthase and modulates its subcellular localization, but the role of NOSTRIN in pathophysiology in vivo is unclear.

Objective: We analyzed the consequences of deleting the NOSTRIN gene in endothelial cells on NO production and cardiovascular function in vivo using NOSTRIN knockout mice.

Methods and Results: The levels of NO and cGMP were significantly reduced in mice with endothelial cell-specific deletion of the NOSTRIN gene resulting in diastolic heart dysfunction. In addition, systemic blood pressure was increased, and myograph measurements indicated an impaired acetylcholine-induced relaxation of isolated aortic rings and resistance arteries. We found that the muscarinic acetylcholine receptor subtype M3 (M3R) interacted directly with NOSTRIN, and the latter was necessary for correct localization of the M3R at the plasma membrane in murine aorta. In the absence of NOSTRIN, the acetylcholine-induced increase in intracellular Ca2+ in primary endothelial cells was abolished. Moreover, the activating phosphorylation and Golgi translocation of endothelial NO synthase in response to the M3R agonist carbachol were diminished.

Conclusions: NOSTRIN is crucial for the localization and function of the M3R and NO production. The loss of NOSTRIN in mice leads to endothelial dysfunction, increased blood pressure, and diastolic heart failure. (Circ Res. 2015;117:460-469. DOI: 10.1161/CIRCRESAHA.115.306187.)

Key Words: echocardiography • GTP-binding proteins • nitric oxide synthase type III • NOSTRIN protein, mouse • receptors, G-protein-coupled

Endothelial cells play a crucial role in controlling vascular tone, thrombosis/fibrinolysis, vascular inflammation, and angiogenesis, as well as regulating cardiomyocyte relaxation. Many of these actions rely on the generation of nitric oxide (NO) as an essential autocrine and paracrine factor.1–4 Reduced activity of the endothelial NO synthase (eNOS) and decreased NO bioavailability are characteristics of endothelial dysfunction, an early event in the development of cardiovascular diseases, such as hypertension, atherosclerosis, and heart failure.5–7

eNOS can be activated in response to numerous agonists, for example, acetylcholine, vascular endothelial growth factor, bradykinin, sphingosine-1-phosphate, or hemodynamic stimuli, such as fluid shear stress. The vessel relaxation elicited by acetylcholine is mediated by the endothelial muscarinic acetylcholine receptor subtype M3 (M3R). This has been demonstrated by comparative analysis of knockout mice for the different muscarinic acetylcholine receptor subtypes M1R–M5R for the aorta,8,9 as well as for smaller arteries, such as pulmonary,10 ophthalmic,11 cutaneous, skeletal muscle, and renal interlobar arteries,12 retinal arterioles,13 and coronary arteries.14 The M3R is a class A, rhodopsin-like, Gαq/11-protein–coupled receptor, and ligand binding leads to increased levels of intracellular Ca2+ ([Ca2+]i) resulting in eNOS activation.

The activity of eNOS is controlled in a complex fashion, including the Ca2+-dependent interaction with calmodulin or other regulatory proteins, reversible phosphorylation, and
the shuttling between distinct subcellular domains.\textsuperscript{2,7,15} eNOS is primarily localized to the plasma membrane and plasma membrane caveolae, as well as to the cytoplasmic face of the Golgi, and these pools have been shown to be connected via a reversible acylation/deacylation cycle.\textsuperscript{16–18} In its inactive state, the caveolar eNOS is associated with caveolin and is thus inhibited. Agonist stimulation induces dissociation of the eNOS/caveolin complex, and enzyme activation seems to be functionally coupled to caveolae internalization and assisted by dynamin.\textsuperscript{2,19,20}

We have previously identified the F-BAR protein NO\textsuperscript{syn} synthase traffic inducer (NOSTRIN) as an eNOS interaction partner.\textsuperscript{2} F-BAR proteins are multivalant adaptors that link the plasma membrane and cytoskeleton, and they are vital for cellular processes, such as membrane protrusion, migration, and endocytosis. The current model for BAR protein function in clathrin-mediated endocytosis suggests that BAR proteins with increasing degree of curvature of their BAR domain are sequentially recruited to and detached from the site of endocytosis as it progresses from shallow pit formation to vesicle scission. The BAR protein function lies in the sculpting of increasing membrane curvature and the recruitment of endocytic proteins, such as the GTPase dynamin, as well as the association with the actin cytoskeleton. Moreover, they can sense membrane curvature through their BAR domain, and hence, they target their binding partners to distinct membrane subdomains or subcellular localization.\textsuperscript{22–25} We have shown that NOSTRIN modulates the subcellular trafficking of eNOS by mediating the interaction between eNOS and the endocytic machinery components dynamin and neuronal Wiskott–Aldrich Syndrome protein,\textsuperscript{21,29,30} hence the name NO\textsuperscript{syn} synthase traffic inducer.\textsuperscript{21,29,31} However, the function of NOSTRIN for regulating NO production in vivo and its role in pathophysiology are unclear to date.

### Methods

#### Mice

Global NOSTRIN knockout (MGI symbol Nostrin\textsuperscript{tm1.1Oess}) and NOSTRIN\textsuperscript{flox/flox} mice (Nostrin\textsuperscript{fl/fl}) were created by targeted mutation using the Cre-lox technology, in the latter case, in combination with Tie2-driven expression of Cre-recombinase as described.\textsuperscript{32} Tie2-Cre mediates excision of pLox site–flanked sequences in endothelial and hematopoietic cells; for simplification, the resulting mice are referred to as endothelial specific. Littermates or age-matched offspring from breedings of Tie2-Cre-positive male with Tie2-Cre-negative female NOSTRIN flox/flox mice were used. Tie2-Cre-negative NOSTRIN flox/flox mice are referred to as control. Mice were backcrossed to C57BL\textsuperscript{6/J}OlaHsd (Harlan Laboratories, Rossdorf, Germany) for 6 generations. Mice were maintained at the animal facility of the Goethe University Frankfurt, Medical School. All animal experiments were performed in compliance with the relevant laws and institutional guidelines and were approved by the animal ethics committee under license number FU/1007. Isolation of murine lung endothelial cells was carried out as described.\textsuperscript{32,33}

#### Phenotypic Characterization

Nitrate/nitrite levels were measured using a colorimetric assay kit (Cayman Chemical Company, Ann Arbor, MI). cGMP concentrations were determined by direct cGMP ELISA (ENZO life science, Lörrach, Germany). High-resolution echocardiography analysis was performed using a Vevo2100-high resolution imaging system (VisualSonics, Toronto, Canada) with a 40-MHz transducer (MS-550D) as described.\textsuperscript{34} Myograph vascular reactivity analysis of thoracic aortae and mesenteric arteries was performed on a DMT 610M small vessel myograph (Danish Myograph Technology, Aarhus, Denmark) as described\textsuperscript{35,36} and analyzed using LabChart 7 software (AdInstruments, Oxford, United Kingdom). Hindlimb perfusion was measured as described.\textsuperscript{37} Systolic blood pressure and heart rate were measured with the noninvasive tail-cuff system (Visitech Systems, Apex, NC). For statistical analysis, GraphPad Prism software (GraphPad Software Inc, La Jolla, CA) was used throughout.

#### Antibodies

Generation of NOSTRIN antibodies was described previously.\textsuperscript{32,38} Additional antibodies were monoclonal eNOS and monoclonal peNOS Ser\textsuperscript{1179} antibody (BD Bioscience, Heidelberg, Germany), polyclonal rabbit anti-M3R antibody (Santa Cruz Biotechnology, Heidelberg, Germany), monoclonal goat anti-platelet endothelial cell adhesion molecule-1 antibody (BD Bioscience, Heidelberg, Germany), polyclonal anti-caveolin-1 antibody (Santa Cruz Biotechnology, Heidelberg, Germany), monoclonal mouse anti-GAPDH antibody (Abcam, Cambridge, United Kingdom), monoclonal anti-extracellular signal-regulated protein kinases 1 and 2 (ERK1/2; Cell Signaling Technology, Leiden, The Netherlands), monoclonal mouse anti-maltose-binding protein (NEB, Ipswich, MA), and polyclonal rabbit anti-green fluorescent protein antibody (Clontech, Saint-Germain-en-Laye, France).

#### Protein Biochemistry and Microscopy Techniques

Purification of glutathione-S-transferase (GST)– and maltose-binding protein–tagged fusion proteins, NOSTRIN–M3R interaction analysis, immunofluorescence staining, microscopy, calcium imaging, and histological analysis were carried out as described in the Online Data Supplement.

### Results

**NO Release and cGMP Production Are Reduced, and Blood Pressure Is Elevated in NOSTRIN Knockout Mice**

To determine whether NOSTRIN plays a role in the regulation of eNOS activity in mice in vivo, the combined levels of NO\textsuperscript{−} and NO\textsuperscript{3−} in the serum of NOSTRIN\textsuperscript{∆EC} mice were measured. A 40% reduction in combined NO\textsuperscript{−} and NO\textsuperscript{3−} levels in the serum was determined as a measure of NO release.

In the serum of NOSTRIN\textsuperscript{∆EC} mice, there was a significant, ≈40% reduction in combined NO\textsuperscript{−} and NO\textsuperscript{3−} levels in comparison with the respective control animals (Figure 1A). Similarly, the levels of cGMP were reduced in lung tissue from NOSTRIN\textsuperscript{∆EC} mice (Figure 1B), an effect that was unrelated to altered expression of the eNOS protein (Figure 1C). This indicated that NOSTRIN regulates the production of NO in vivo. To determine whether the impaired NO and cGMP production translated into a hypertension phenotype, blood pressure was measured in male NOSTRIN\textsuperscript{∆EC} mice at an age of 8 to 10 weeks. The endothelial cell–specific deletion of NOSTRIN led to a significant increase in systolic blood pressure when compared with controls (Figure 1D). These changes in blood
NOSTRIN cGMP production and causes elevated blood pressure in Loss of NOSTRIN impairs NO release and

Figure 1. (E) and late atrial (A) diastolic velocities in NOSTRIN Doppler revealed a significant reduction of the ratio of early (E) and late (A′) diastolic mitral annulus velocities determined by tissue Doppler imaging was reduced (Figure 2C and 2D), and the
e/E′ ratio was significantly increased in NOSTRINEC mice (Figure 2E). The isovolumic relaxation time was significantly increased in NOSTRINEC mice (Figure 2F), and the myocardial performance index was likewise increased (Figure 2G).

Taken together, the Doppler echocardiography revealed the evidence of a significant diastolic dysfunction in NOSTRINEC mice. There were no differences in cardiac output and cardiac index, indicating that the systolic heart function was normal in NOSTRINEC mice (Figure 2H; Online Figure IF). Moreover, there was no evidence of cardiac hypertrophy (Online Figure IA–IN).

Acetylcholine-Induced, NO-Dependent Relaxation Is Impaired in Aortic Rings From NOSTRINEC Mice

Acetylcholine-induced relaxation of aortic rings isolated from NOSTRINEC mice was impaired (Figure 3A) and accompanied by a significant increase in the $E_{C50}$ from 3.01 μmol/L in control mice to 60.38 μmol/L ($P<0.0001$; $n=23$) in NOSTRINEC mice. In the same animals, neither the vasoconstrictor response to the agonist phenylephrine (Figure 3B) nor the vasorelaxation to sodium nitroprusside (Figure 3C) was influenced by the deletion of NOSTRIN.

Next, responses to a second endothelial agonist, that is, the α2-agonist UK14304, were assessed. In aortic rings isolated from NOSTRINEC mice, the relaxation response to UK14304 was comparable with that of aortic rings from control mice (Figure 3D). In addition, the application of the calcium ionophore A23187 induced comparable relaxations in NOSTRINEC and control mice (Figure 3E). Therefore, of the agents tested, the inhibitory effect of the loss of NOSTRIN on endothelium-dependent relaxation seemed to be restricted to acetylcholine (Figure 3F).

In addition to NO, vessel relaxation can be achieved through the action of other endothelium-derived relaxing factors. To determine the dependence of the acetylcholine-induced relaxation responses on prostacyclin, experiments were repeated in the presence of the cyclooxygenase inhibitor diclofenac. Cyclooxygenase inhibition failed to affect the attenuated acetylcholine response of aortic rings from NOSTRINEC mice (Figure 4A). As acetylcholine-induced NO-mediated relaxation could be affected by the enhanced production of reactive oxygen species, responses were assessed in the absence and presence of catalase and the superoxide dismutase mimetic, tempol. Neither catalase (to target $H_2O_2$) nor tempol (to target $O_2^-$) altered the responses of aortic rings from either control or NOSTRINEC mice (Figure 4B and 4C). In contrast, addition of the NOS inhibitor L-NG-nitroarginine methyl ester completely prevented acetylcholine-induced relaxation (Figure 4B and 4C), indicating that the loss of NOSTRIN primarily affects the production of NO and that alterations in prostacyclin, cyclooxygenase-derived endothelium-dependent contracting factors, or reactive oxygen species levels cannot account for the phenotype observed in the NOSTRINEC mice. Similarly, we failed to observe significant differences in the production of reactive oxygen species from aorta (Online Figure IIA) or altered protein levels of catalase and superoxide dismutase in primary endothelial cells or the left ventricles from control and NOSTRINEC mice (Online Figure IIB and IIC).
In the Absence of NOSTRIN, Relaxation of Resistance Arteries and Vasodilatation in the Hindlimb Are Impaired

Studying responses in aortic rings has the advantage that NO is the primary vasodilator; however, the aorta is a conductance vessel that plays no role in the determination of vascular resistance. Therefore, NO-mediated relaxation was also assessed in isolated second order mesenteric resistance arteries. As in the aortic rings, acetylcholine-induced relaxation was significantly impaired in mesenteric arteries isolated from NOSTRIN\textsuperscript{ΔEC} mice in comparison with control mice (Figure 4D). Also, in a more physiological situation, that is, the perfused hindlimb comprising an entire vascular bed,\textsuperscript{37} acetylcholine-induced vasodilatation was significantly impaired in the absence of NOSTRIN (Figure 4E). The changes in vascular reactivity were not associated with gross morphological changes, that is, in the aorta or the mesenteric arteries (Figure 4F). Collectively, these data indicate that NOSTRIN is involved in NO-dependent vasorelaxation in conductance and resistance vessels.

NOSTRIN Is Necessary for the Correct Spatial Localization of the M3R in Mouse Aorta

Given that the impaired relaxation of aortic rings from NOSTRIN\textsuperscript{ΔEC} mice was restricted to those stimulated with acetylcholine, we hypothesized that the loss of NOSTRIN might directly affect the function of the endothelial acetylcholine receptor M3R. The loss of NOSTRIN had no influence on protein levels of the M3R (Figure 5A) or eNOS (Figure 5B) in murine aortae. This confirmed the previous observations in the lung (Figure 1C) and ruled out that the differences in vasorelaxation stemmed from altered eNOS expression. However, en face immunofluorescence staining revealed that although the M3R was localized at endothelial cell/cell contacts in aortae from control mice, this subcellular localization was lost in NOSTRIN\textsuperscript{ΔEC} mice, where M3R staining was restricted to intracellular punctae (Figure 5C).

NOSTRIN Interacts Directly With the Third Intracellular Loop of the M3R

To determine whether an interaction exists between the M3R and NOSTRIN, M3R–green fluorescent protein and NOSTRIN–mCherry fusion proteins were expressed in U2OS cells. Both proteins colocalized at the cell border in unstimulated cells and preferentially to intracellular punctae after stimulation with carbachol (Figure 6A). Furthermore, in a GST-pulldown assay, GST–NOSTRIN interacted with M3R–green fluorescent protein expressed in U2OS cells. A mutant of NOSTRIN lacking the SH3 domain (GST–NOSTRIN–ΔSH3) failed to interact, indicating that the SH3 domain is necessary for the interaction. However, the SH3 domain alone (GST–SH3) was not sufficient to mediate the interaction with the M3R (Figure 6B). Next, GST-pulldown experiments using
the GST–NOSTRIN fusion proteins were performed in combination with a purified fusion protein of the third intracellular loop of the M3R (i3loop; amino acids 253–492) with an N-terminal maltose-binding protein tag. As before, the full-size NOSTRIN interacted with the i3loop of the M3R, and this direct interaction was abolished after deletion of the SH3 domain. However, the SH3 domain alone was not sufficient to interact with the M3R i3loop (Figure 6C).

**NOSTRIN Is Necessary for the Acetylcholine-Induced Ca2+ Signal in Primary Endothelial Cells**

The M3R is coupled to Gαq/11, and in response to ligand binding, it activates phospholipase Cβ leading to the mobilization of intracellular Ca2+. Therefore, Ca2+ was assessed using the Ca2+ indicator Fura-2 in primary endothelial cells. After the application of acetylcholine (10 μmol/L), the mean normalized Fura-2 $F_{340}/F_{380}$ ratio was significantly increased in wild-type but not in NOSTRIN-deficient endothelial cells (Figure 7A). Because endothelial cells and aortic endothelium have been reported to respond heterogeneously to acetylcholine and this has been attributed to the heterogeneous expression pattern of the M3R, the number of acetylcholine-responsive cells in wild-type and NOSTRIN-deficient cells was compared. After the application of acetylcholine (10 μmol/L), 48.1% of the wild-type endothelial cells responded with a Ca2+ peak, whereas only 1.5% of NOSTRIN-deficient endothelial cells responded. Analysis of the Ca2+ response in a single wild-type endothelial cell with a typical acetylcholine response revealed a characteristic rapid peak followed by a plateau phase, whereas typically no response could be observed in NOSTRIN-deficient endothelial cells (Figure 7B).

**Carbachol Fails to Activate eNOS in the Absence of NOSTRIN**

Activation of eNOS is accompanied by its translocation to the Golgi complex and the phosphorylation at activating...
residues, for example, Ser1179. When the proportion of eNOS localized to the Golgi complex was compared in aortae from NOSTRINΔEC mice versus their respective controls (Figure 8A), a significant reduction of the eNOS-positive Golgi area was detected (Figure 8B). Accordingly, the level of Ser1179-phosphorylated eNOS was reduced in aortic tissue lysates from NOSTRINΔEC mice (Figure 8C). The expression of caveolin-1 was not affected by the loss of NOSTRIN (Figure 8D). Finally, the carbachol-induced phosphorylation of eNOS on Ser1179 at the Golgi complex was impaired in NOSTRIN-deficient endothelial cells (Figure 8E), consistent with an impaired activation of eNOS in the absence of NOSTRIN.

Discussion

The results of this study indicate that NOSTRIN interacts directly with the M3R and is required for its correct spatial localization at the plasma membrane in aortic endothelial cells. In the absence of NOSTRIN, the function of the M3R was markedly impaired, resulting in abolition of the calcium response to acetylcholine, an impaired activation of eNOS, and the inhibition of vascular relaxation. These changes at the cellular level were reflected in vivo in the existence of elevated blood pressure and diastolic dysfunction in NOSTRINΔEC mice.

The interaction of NOSTRIN with the M3R is to our knowledge the first example of an F-BAR protein interacting with a Gαq/protein–coupled receptor. The interaction is direct and involves the SH3 domain of NOSTRIN. The SH3 domain alone was not sufficient to bind to the M3R, indicating that additional motifs in NOSTRIN might be required. On the side of the M3R, the interaction with NOSTRIN involved the i3loop, which comprises 240 amino acids but contains no canonical SH3-binding proline-rich motif, indicating that nonconsensus and atypical SH3 domain ligands might be responsible for the binding to the NOSTRIN SH3 domain.

The correct spatial localization of the M3R at the endothelial cell membrane of the mouse aorta is lost in the absence of NOSTRIN, indicating that NOSTRIN might be involved in the recycling of the M3R. This is supported by the fact that the i3loop, the site of NOSTRIN interaction, has a recognized role in receptor regulation, for example, in G-protein coupling and the interaction with G-protein–coupled receptor kinase-2 and β-arrestin. The molecular details of M3R recycling in...
the cardiovascular system are unclear to date, but overexpression studies in other cell types indicate constitutive, clathrin-independent recycling through the endosomal pathway and an agonist-induced switch to clathrin-dependent endocytosis that is dependent on the i3loop. In general, F-BAR proteins like other members of the BAR protein superfamily have been shown to be involved both in clathrin-dependent and clathrin-independent endocytosis. In addition, BAR proteins of the SNX-BAR family have been reported to form part of the retromer complex, which is critically involved in the endosomal sorting and recycling of membrane-associated cargo proteins. Therefore, it is conceivable that NOSTRIN could be involved in the trafficking of the M3R at several of the discrete steps of receptor recycling. In line with this notion, it has been shown that NOSTRIN localizes to rab11-positive (and to a lesser extend rab5-positive) endosomes and might function at endosomal tubule intermediates to mediate microtubule-dependent trafficking of recycling endosomes.

We originally identified NOSTRIN as an eNOS interaction partner and established its function as an adaptor for eNOS, dynamin, neuronal Wiskott–Aldrich Syndrome protein, and caveolin that affected the subcellular localization of eNOS. However, the role of NOSTRIN in regulating NO production in vivo and its role in pathophysiology were unclear. Here, we show that in the absence of NOSTRIN, eNOS activity was compromised, evident as impaired Golgi translocation and phosphorylation at the activating Ser1179 site. This is highly consistent with the impaired acetylcholine-induced relaxation of isolated aortic rings and mesenteric arteries, as well as the compromised acetylcholine-induced vasodilatation of the hindlimb vasculature, confirming the role of NOSTRIN as an important regulator of eNOS function. However, it was unexpected that the inhibition of vasorelaxation in the absence of NOSTRIN occurred not generally in response to eNOS-activating stimuli but specifically on acetylcholine stimulation (of the reagents we have tested). This suggests that NOSTRIN is not generally necessary for the activity of eNOS but rather is involved in the activation in response to specific signals, such as acetylcholine. This would be in line with the concept that specific portions of the cellular eNOS pool are preassembled into larger signaling complexes, allowing for controlled spatial activation or local NO production.

Although endothelial cells express the M3R and acetylcholine is routinely used in in vitro studies to assess endothelial cell function and NO production, there is considerable debate about the in vivo relevance of acetylcholine for the regulation of vascular tone and resistance. Indeed, acetylcholine is produced by perivascular nerve fibers, but they are restricted to some arterial vascular beds. Rather, endothelial cells have been shown to serve as the endogenous source for acetylcholine, and for example, in the coronary vasculature, they generate the transmitter in sufficient amounts to induce NO release. Such observations argue in favor of an important physiological role of acetylcholine in the regulation of vascular tone. The question which aspects of the phenotype in NOSTRIN mice are dependent on the impaired function of acetylcholine and which might be independent is difficult to answer unambiguously because knockout mice with endothelial-cell specific deletion of the M3R are not available for comparison.

In this study, we show that NOSTRIN mice exhibit elevated blood pressure, and this is in line with the important function of NO as a mediator of vessel relaxation. In accordance with our results, hypertension is a hallmark characteristic in eNOS knockout mice. The diastolic dysfunction in NOSTRIN mice is indicated by decreased left ventricular E/A and E’/A’ ratios, an increased E/E’ ratio, and an increased isovolumic relaxation time and myocardial performance index, whereas the ejection fraction was preserved. Collectively, these parameters describe an impaired active cardiac relaxation and increased stiffness. The existence of diastolic dysfunction in NOSTRIN mice can be explained by the recognized role of endothelial-derived NO in promoting cardiomyocyte relaxation. However, the phenotype is more severe than in eNOS knockout mice, where additional stresses are necessary to evoke diastolic dysfunction, for example, chronic pressure overload or the simultaneous deletion of the 2 other NOS
isoforms neuronal NO synthase and inducible NO synthase. This indicates that NOSTRIN has additional roles or binding partners, which might contribute to the cardiovascular phenotype in NOSTRIN\(\Delta EC\) mice.

In humans, diastolic dysfunction often precedes diastolic heart failure or heart failure with preserved ejection fraction, which is a major cause of mortality in the elderly population. In contrast to heart failure with reduced ejection fraction, which includes systolic dysfunction and where the identification of key pathological mechanisms led to the development of targeted pharmacological interventions and improvement in disease prognosis, the pathophysiological mechanisms underlying heart failure with preserved ejection fraction are far from being understood. In humans, the NOSTRIN gene is located on chromosome 2 on position 2q31.1. A region encompassing the NOSTRIN gene (2q24.3–2q31.1) has been identified as a novel locus for hypertension in a Kyrgyz family, pointing toward a possible role of NOSTRIN as an important pathophysiological factor.

Figure 8. eNOS activation is impaired in the absence of NOSTRIN. A. Confocal immunofluorescence microscopy images of en face stained aorta explants from mice of the indicated genotype using an eNOS- or Giantin-specific antibody. B. Quantification of eNOS-labeled Golgi complex as shown in (A). C. Immunoblot of aortae from mice of indicated genotype with peNOS\(1179\) and eNOS-specific antibodies. D. Immunoblot of primary murine lung endothelial cells (MLECs) from mice of indicated genotype with eNOS- and caveolin-1-specific antibodies. E. Confocal immunofluorescence microscopy images of primary MLECs from mice of indicated genotype with peNOS\(1179\)-specific antibody in combination with DAPI unstimulated or after stimulation with the M3R agonist carbachol (100 \(\mu\)mol/L; 5 minutes). \(\Delta EC\) indicates NOSTRIN\(\Delta EC\); CTL, control; KO, knockout; NOSTRIN, nitric oxide synthase traffic inducer; and WT, wild-type.
Acknowledgments

We thank Dr K. Scholich and S. Hohmann, Institut für Klinische Pharmakologie, Klinikum der Goethe-Universität Frankfurt, Germany, for support with Ca2+ measurements.

SOURCES OF FUNDING

This work was supported by the German research foundation, Deutsche Forschungsgemeinschaft, SFB 834 project A3 to S. Oess and project A9 to I. Fleming and Excellence Cluster Cardio Pulmonary Systems (ECCPS) EXC 147 to R.T. Schermuly.

Disclosures

None.

References

Novelty and Significance

What Is Known?

- Endothelial-derived nitric oxide (NO) mediates vasodilatation and cardiomyocyte relaxation, and reduced NO production is associated with cardiovascular disease.
- NO synthase traffic inducer (NOSTRIN) interacts with endothelial NO synthase and modulates its intracellular localization.
- NOSTRIN belongs to the F-BAR/BAR protein family, whose members have important functions in membrane remodeling during endocytosis and vesicular traffic.

What New Information Does Your Article Contribute?

- NO production is impaired in mice with endothelial-cell specific deletion of the NOSTRIN gene (NOSTRIN−/− mice).
- NOSTRIN−/− mice show diastolic heart dysfunction, increased blood pressure, and an impaired acetylcholine-induced relaxation of conductance and resistance arteries.
- NOSTRIN interacts directly with the muscarinic acetylcholine receptor subtype M3 and is crucial for its correct localization and function.

This study was conducted to determine the role of NOSTRIN in cardiovascular pathophysiology. In the absence of NOSTRIN, the function of the muscarinic acetylcholine receptor subtype M3 was markedly impaired, resulting in abolition of the calcium response to acetylcholine, an impaired activation of endothelial NO synthase, and the inhibition of vascular relaxation. These changes were reflected in vivo in the existence of elevated blood pressure by chronic pressure overload. Cardiovasc Res. 2005;66:444–453. doi: 10.1016/j.cardiores.2005.01.021.

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Circ Res. 2015;117:460-469; originally published online July 13, 2015;
doi: 10.1161/CIRCRESAHA.115.306187

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/117/5/460

Data Supplement (unedited) at:
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SUPPLEMENTAL MATERIAL

DETAILED METHODS

Serum nitrate/nitrite, cGMP concentration measurement
For the assessment of NO production nitrate/nitrite levels in blood serum of 8-10 week old mice were measured using a colorimetric assay kit (Cayman Chemical Company, Ann Arbor, Michigan USA). Mice were anaesthetised with isoflurane (Forene®) and sacrificed by cervical dislocation and blood was withdrawn from the heart using a 23G syringe. Upon clotting, blood samples were centrifuged 5 min at 2,400 g and supernatant was filtered using centrifugal filters with 30,000 MWCO (Amicon; Merck Millipore, Schwalbach, Germany). 40 µl of filtered serum was assayed for the nitrate/nitrite concentration according to the manufacturer’s instructions. The values were calculated by measuring the absorbance at 540 nm using a VICTOR fluorescence plate reader (PerkinElmer, Waltham, Massachusetts, USA). Statistical analysis from n ≥ 3 experiments was done by unpaired two-tailed t-test using GraphPad Prism software (GraphPad Software Inc, La Jolla, California, USA).

The analysis of the cGMP concentration was performed from lung tissues isolated from 8-10 week old mice. Freshly isolated lungs were frozen in liquid nitrogen, ground to a powder and weighed. Tissue was lysed in 10 volumes of 100 mmol/L HCl, centrifuged 5 min at 16,000 g at room temperature and the supernatant was used for the measurement of the cGMP concentration using a direct cGMP ELISA kit (ENZO life science, Lörrach, Germany) according to manufacturer’s instructions. The reaction was stopped by addition of stop solution and absorbance was read at λ405 nm using the VICTOR fluorescence plate reader (PerkinElmer, Waltham, Massachusetts, USA). Data are shown as relative cGMP concentrations normalised to samples obtained from control mice. Statistical analysis from n ≥ 3 experiments was done by column statistics/t-test using GraphPad Prism software (GraphPad Software Inc, La Jolla, California, USA).

Measurement of hydrogen peroxide production in aorta explants
For the measurement of hydrogen peroxide production 8-10 week old control or NOSTRIN∆EC mice were anaesthetised with isoflurane (Forene®) and sacrificed by decapitation. Thoracic aortae were isolated, cleaned of excessive fat tissue and cut longitudinally into two equal halves. One half was incubated with catalase (50 U/µl) (Sigma, Taufkirchen, Germany). Peroxide production measurement was performed using the PeroxiDetect™ kit (Sigma, Taufkirchen, Germany) according to the manufacturer’s instructions. Hydrogen peroxide levels were calculated as difference of catalase treated and untreated samples. Statistical analysis from n = 9 experiments was done by unpaired two-tailed t-test using GraphPad Prism software (GraphPad Software Inc, La Jolla, California, USA).

High resolution echocardiography analysis
Transthoracic two-dimensional, M-mode and Doppler imaging were performed on 8-10 week old control or NOSTRIN∆EC mice using a Vevo2100-high resolution imaging system (VisualSonics, Toronto, Canada) with a 40-MHz transducer (MS-550D) (VisualSonics, Toronto, Canada). Anaesthesia was induced with 3% isoflurane (Forene®) and maintained with 1.0–1.5% isoflurane (Forene®) in 100% O2. Mice were laid supine on a heating platform with all legs taped to ECG electrodes for heart rate monitoring. Body temperature was monitored via a rectal thermometer (Indus Instruments, Houston, TX, USA) and maintained at 36.5–37.5°C using a heating pad and an infrared lamp. The chest of the mice was shaved and treated with a chemical hair remover to reduce ultrasound attenuation. To provide a coupling medium for the transducer, a pre-warmed ultrasound gel was spread over the chest wall. Measurements were performed on at least 6 animals per group. Statistical analysis was done by unpaired two-tailed t-test using GraphPad Prism software (GraphPad Software Inc, La Jolla, California, USA).
**Myograph vascular reactivity analysis**

For the myograph analysis, vessels were isolated from 8-10 week old control or NOSTRIN^{AE}C mice. Animals were anaesthetised with isoflurane (Forene®) and sacrificed by decapitation. Thoracic aortae were isolated, cleaned of excessive fat tissue and cut into 2 mm wide rings. The experiments were performed on a DMT 610M small vessel myograph (Danish Myograph Technology, Aarhus, Denmark) as previously described 1. In brief, the relaxation of the aortic rings pre-contracted with phenylephrine was assessed in response to increasing concentrations of pharmacological compounds as indicated. The concentration of phenylephrine, used for pre-contraction, was adjusted to obtain an identical pre-contraction level of 80% of the concentration elicited by KCl (80 mmol/L), which was usually around 0.8 g. Obtained data were analysed using LabChart 7 software (AdInstruments, Oxford, United Kingdom). Statistical analysis was done by two-way ANOVA using GraphPad Prism software (GraphPad Software Inc, La Jolla, California, USA).

Branch II of resistance arteries were cleaned of fat and connective tissue and cut into 2 mm long segments. The experiments were performed on a DMT 610M small vessel myograph (Danish Myograph Technology, Aarhus, Denmark) as previously described 2. Briefly, two tungsten wires (15 µm diameter) were inserted through the lumen of the vessel. The vessel was equilibrated for 30 min before being passively stretched to an internal diameter that yields a circumference equivalent to 90% of that given by an internal pressure of 100 mmHg, which requires a load of 250 mg. The internal diameter of the vessels used in this study ranged between 100 and 150 µm. After the vessel was set to its working length, it was challenged twice with KCl (80 mmol/L) to elicit reproducible contractile responses. Measurements were performed on ≥ 3 animals and obtained data were analysed using LabChart 7 software (AdInstruments, Oxford, United Kingdom). Statistical analysis was done by two-way ANOVA with Bonferroni post test using GraphPad Prism software (GraphPad Software Inc, La Jolla, California, USA).

**Perfused hindlimb**

Acetylcholine-induced vasodilator responses of the mouse hindlimb were measured in 8-10 week old control or NOSTRIN^{AE}C mice as changes in perfusion pressure as described previously 3. Briefly, mice were euthanized, under anaesthesia with 2.5% isoflurane (Forene®) by a transverse cut through the large abdominal vessels, before a Teflon i.v. catheter (0.67 mm, 24 gauge, Durflon; Terumo, Leuven, Belgium) was introduced into the aorta, advanced to one of the iliac arteries and tied. Venous congestion was avoided by cutting the inferior caval vein. The hindlimb was permanently perfused with modified filtered Krebs–Henseleit solution (NaCl 118.3 mmol/L; KCl 4.7 mmol/L; CaCl2 1.8 mmol/L, MgSO4 1.2 mmol/L, KH2PO4 1.2 mmol/L, NaHCO3 25 mmol/L, EDTA 0.026 mmol/L, glucose 11.1 mmol/L; pH 7.4 aerated with 95% O2/5% CO2 using a roller pump, whereas a pressure transducer and a compliance chamber were connected to one side part of the system. Flow rate was gradually increased up to a corresponding perfusion pressure of around 100 mmHg, where a considerable amount of spontaneous myogenic tone is present and further pre-contraction can be avoided. When a stable pressure plateau was reached, acetylcholine was applied in increasing concentrations as a bolus (100 µL) in glucose solution (50 g/L). Agonist-induced vasodilatations were calculated as changes in perfusion pressure. Measurements were performed on 6 animals. Statistical analysis was done by two-way ANOVA with Bonferroni post test using GraphPad Prism software (GraphPad Software Inc, La Jolla, California, USA).

**Blood pressure and heart rate measurement**

Systolic blood pressure and heart rate were measured in adult 8-10 week old mice using non-invasive tail-cuff system (Visitech Systems, Apex, North Carolina, USA). In order to minimise stress-induced blood pressure changes, animals were trained for five consecutive days and only the blood pressure value measured on the fifth day was included in the analysis. The analysis was performed on 6-8 mice per group. Statistical analysis was done
by unpaired two-tailed t-test using GraphPad Prism software (GraphPad Software Inc, La Jolla, California, USA).

Isolation of mouse lung endothelial cells
Isolation of MLECs from NOSTRIN KO and WT mice for immunofluorescence analysis of eNOS Ser\(^{1179}\) phosphorylation was carried out as described\(^4\). Endothelial cells were isolated from lungs of 3-6 pups at postnatal day (P)14-P7 and cells were seeded on gelatine-coated glass coverslips. MLECs in passage 2 were used for the immunofluorescence analysis of eNOS Ser\(^{1179}\) phosphorylation. For immunoblot analysis and calcium imaging MLECs were isolated from lungs of 8-10 week old mice according to\(^5\). Cells were seeded on fibronectin-coated glass coverslips and used for calcium imaging at passage 0.

Purification of GST- and MBP-tag fusion proteins
GST-fusion proteins of NOSTRIN and NOSTRIN deletion constructs were purified on glutathione (GSH)-Sepharose (GE Healthcare, Freiburg, Germany) as previously described\(^6\). Full-length human M3 muscarinic acetylcholine receptor cDNA was obtained from Fermentas (Fermentas, St. Leon-Rot, Germany). i3loop (aa253-492) of the human acetylcholine M3R was cloned into the pMALc2x vector (NEB, Ipswich, Massachusetts, USA). Resulting MBP-tagged fusion protein was purified from \(E.\ coli\) BL21 using amylose resin (NEB, Ipswich, Massachusetts, USA) according to the manufacturer’s instructions.

NOSTRIN-M3R interaction analysis
For the pulldown experiments lungs from 8-10 week old WT mice were isolated, minced in liquid nitrogen and homogenized with a Dounce homogenizer in lysis buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 2 mmol/L EDTA, 1 % NP40 supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany), 1 mmol/L PMSF, phosphatase inhibitors (25 mol/L NaF and 1 mmol/L Na\(_3\)VO\(_4\)) and 10 U/ml benzonase). Lysates were incubated overnight at 4°C with GSH-sepharose beads coupled with equal amounts of GST-tagged NOSTRIN or GST–NOSTRIN-\(\Delta\)-SH3 or GST-NOSTRIN-SH3. Subsequently, beads were washed three times with lysis buffer and boiled in protein sample buffer (63 mmol/L Tris-HCl pH 6.8, 5 % (w/v) glycerol, 5 % (w/w) \(\beta\)-mercaptoethanol, 2.5 % (w/v) SDS, 0.005 % bromophenol blue). Bound proteins were resolved in SDS-PAGE followed by immunoblotting with polyclonal anti-M3R antibody (Santa Cruz Biotechnology, Heidelberg, Germany). For the pulldowns with GFP-tagged M3R, lysates were prepared from stable U2OS cell lines expressing M3R-GFP. The pulldown procedure was as above and proteins were immunoblotted using polyclonal anti-GFP antibody (Clontech, Saint-Germain-en-Laye, France). For the direct protein interaction studies between NOSTRIN and the i3loop of M3R, equal amounts of purified MBP-tagged aa253-492 of human M3R were incubated with GSH-sepharose beads coupled with equal amounts of GST-tagged NOSTRIN or GST–NOSTRIN-\(\Delta\)-SH3 or GST-NOSTRIN-SH3 for 2 hours at 4°C in direct pulldown incubation buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 10 % glycerol, 0.1 % BSA, 0.5 % Triton-X-100 supplemented with 1 mmol/L PMSF, 10 \(\mu\)mol/L aprotinin and 1 mmol/L Benzamidine). Subsequently, sepharose beads were washed three times with direct pulldown incubation buffer and two times with PBS. Bound proteins were further analysed by SDS-PAGE and immunoblotting with MBP antibody (NEB, Ipswich, Massachusetts, USA).

Immunofluorescence staining of mouse aorta, primary MLECs and M3R-GFP stable cell lines
Eight -10 week old mice were anaesthetised with isoflurane (Forene\(^6\)) and sacrificed by decapitation. The thoracic aorta was removed and trimmed off fat and excess tissue in cold PBS. The isolated aorta was cut into stripes and fixed with 4 % paraformaldehyde (PFA) in PBS for 10 minutes at 4°C. Subsequently the aorta was permeabilized in 0.3 % Triton-X-100 in PBS for 30 minutes at room temperature and blocked in 5 % BSA in PBS for 1 hour at room temperature. The pieces of aorta were then incubated with primary antibodies
overnight at 4°C with gentle shaking. After washing with PBS, samples were incubated with Cy3-, Cy5- and FITC- conjugated secondary antibodies obtained from Jackson Immunoresearch (West Grove, Pennsylvania, USA) and DAPI, washed with PBS and mounted in 50% glycerol in PBS. Fluorescent images were acquired using the Leica TCS SP5 confocal microscope (Leica, Wetzlar, Germany).

For the immunofluorescence staining of primary mouse lung endothelial cells, cells isolated from WT or NOSTRIN KO mice were stimulated with 100 μmol/L carbachol for 5 min. Cells were fixed with 4% PFA for 15 min at 4°C, permeabilised with 50 μmol/L digitonin for 5 min at room temperature and blocked in 1% BSA in PBS. Primary antibody incubation was performed at 1:50 dilution in blocking buffer, followed by washing and secondary antibody and DAPI incubation as described above. Coverslips were mounted in Mowiol (Carl Roth, Karlsruhe, Germany) and analysed using the Leica TCS SP5 confocal microscope (Leica, Wetzlar, Germany). M3R-GFP construct was cloned into pRESpuro2 vector (Clontech, Saint-Germain-en-Laye, France) and transfected into U2OS cells using GeneJuice® (Merck, Schwalbach, Germany) according to manufacturer’s instructions. Stably transfected U2OS cells were selected using 0.5 μg/ml puromycin. For colocalisation studies NOSTRIN was cloned into pRESpuro2 vector (Clontech, Saint-Germain-en-Laye, France) with C-terminal mCherry-tag. Stable M3R-GFP U2OS cells were seeded on glass coverslips, transfected with NOSTRIN-mCherry-pRESpuro2 using GeneJuice® (Merck, Schwalbach, Germany). 18 hours after transfection cells were fixed with 4% PFA, mounted in Mowiol (Carl Roth, Karlsruhe, Germany) and analysed using the Leica TCS SP5 confocal microscope (Leica, Wetzlar, Germany).

**Immunoblotting**

Immunoblotting was performed using polyclonal anti-catalase antibody (Santa Cruz, Heidelberg, Germany), polyclonal anti-superoxide dismutase antibody (Merck, Schwalbach, Germany).

**Histology**

Histology images were taken on an Olympus BX41 microscope (Olympus, Hamburg, Germany).

**Calcium imaging**

Calcium imaging was performed on lung endothelial cells freshly isolated from adult WT or NOSTRIN KO mice according to a method previously described. Prior to calcium imaging endothelial cells were loaded with 5 μmol/L Fura-2-acetoxymethyl-ester (life technologies, Darmstadt, Germany) for 2 hours at 37°C. Upon incubation cells were rinsed in Ringer solution (10 mmol/L HEPES pH 7.3, 145 mmol/L NaCl, 1.25 mmol/L CaCl2, 1 mmol/L MgCl2, 5 mmol/L KCl, 10 mmol/L glucose) and placed into the bath chamber of the Leica calcium-imaging setup equipped with Leica DMI 4000 b inverted microscope and DFC360 FX (CCD) camera (Leica, Wetzlar, Germany). Ringer solution was perfused into the chamber at a flow rate of 1–2 ml/min. After 1 min the chamber was perfused with Ringer solution containing either 10 μmol/L acetylcholine or 2 μmol/L A23187 calcium ionophore, following 4 min perfusion with empty Ringer solution. During this time course the cells were excited alternately at λ340 or 380 nm and fluorescent images were acquired every 2 seconds at λ515 nm emission. Ratios of the 340/380 nm excited fluorescence intensities were calculated for each cell in the acquired image using LAS AF-software (Leica, Wetzlar, Germany) and displayed as Fura-2-AM 340/380 ratio normalised to the ratio at the beginning of experiment.
Loss of NOSTRIN is not associated with cardiac hypertrophy in mice

(A) Left ventricular fractional shortening. (B) Left ventricular mass/body weight. (C) Interventricular septum thickness. (D) Left ventricular inner diameter in diastole. (E) Left ventricular inner diameter in systole. (F) Cardiac index in mice of the indicated genotype. All measurements were performed in male mice at an age of 8-10 weeks, n ≥6. (G-N) Representative transverse sections of the heart with Martius Yellow stain, showing cardiomyocytes (red), erythrocytes (yellow) and collagen (blue) of mice of the indicated genotype. H (septum) and I (left ventricular wall, LVW) correspond to the boxed areas in G; L (septum) and M (LVW) correspond to the boxed areas in K; J and N show higher magnifications of the LVW. Bars indicate 2000 μm (G/K), 500 μm (H, I, L, M) or 200 μm (J, N), respectively.
Online Figure II

(A) Peroxide production in murine aorta from NOSTRIN knockout (KO) and wildtype (WT) mice. (B) Immunoblot of primary mouse lung endothelial cells generated from mice with the indicated genotype with catalase-, SOD- and NOSTRIN-specific antibodies. Immunoblot with a GAPDH-specific antibody served as loading control. The asterisk indicates an unspecific band detected with the NOSTRIN antibody. (C) Immunoblot of lysates generated from the left ventricle of the heart of mice with the indicated genotype with catalase-, SOD- and NOSTRIN-specific antibodies. Immunoblot with a GAPDH-specific antibody served as loading control. The asterisk indicates an unspecific band detected with the NOSTRIN antibody.
Supplemental References


