Dynamin-2 is a novel NOS1β interacting protein and negative regulator in the collecting duct

Kelly A. Hyndman, Alexandra M. Arguello, Sophia K. H. Morsing, and Jennifer S. Pollock

Section of Cardio-Renal Physiology and Medicine, Division of Nephrology, Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama

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Hyndman KA, Arguello AM, Morsing SK, Pollock JS. Dynamin-2 is a novel NOS1β interacting protein and negative regulator in the collecting duct. Am J Physiol Regul Integr Comp Physiol 310: R570–R577, 2016. First published January 20, 2016; doi:10.1152/ajpregu.00008.2015.—Nitric oxide synthase 1 (NOS1)-derived nitric oxide (NO) production in collecting ducts is critical for maintaining fluid-electrolyte balance. Rat collecting ducts express both the full-length NOS1α and its truncated variant NOS1β, while NOS1β predominates in mouse collecting ducts. We reported that dynamin-2 (DNM2), a protein involved in excising vesicles from the plasma membrane, and NOS1α form a protein-protein interaction that promotes NO production in rat collecting ducts. NOS1β was found to be highly expressed in human renal cortical/medullary samples; hence, we tested the hypothesis that DNM2 is a positive regulator of NOS1β-derived NO production. COS7 and mouse inner medullary collecting duct-3 (mIMCD3) cells were transfected with NOS1β and/or DNM2. Coimmunoprecipitation experiments show that NOS1β and DNM2 formed a protein-protein interaction. DNM2 overexpression decreased nitrite production (index of NO) in both COS7 and mIMCD-3 cells by 50–75%. mIMCD-3 cells treated with a panel of dynamin inhibitors or DNM2 siRNA displayed increased nitrite production. To elucidate the physiological significance of IMCD DNM2/NOS1β regulation in vivo, flox control and CDNOS1 knockout mice were placed on a high-salt diet, and freshly isolated IMCDs were treated acutely with a dynamin inhibitor. Dynamin inhibition increased nitrite production by IMCDs from flox mice. This response was blunted (but not abolished) in collecting duct-specific NOS1 knockout mice, suggesting that DNM2 also negatively regulates NOS3 in the mouse IMCD. We conclude that DNM2 is a novel negative regulator of NO production in mouse collecting ducts. We propose that DNM2 acts as a “break” to prevent excess or potentially toxic NO levels under high-salt conditions.

IN THE COLLECTING DUCT, a number of paracrine, autocrine, and endocrine factors are known to balance sodium and water intake with excretion to maintain homeostasis. The nitric oxide (NO) pathway functions as a natriuretic and diuretic factor. NO is produced by the NO synthase (NOS) family of enzymes and expressed throughout the kidney with the inner medullary collecting ducts (IMCD) having the greatest NOS activity (33). NOS1 (neuronal NOS, or nNOS) is highly expressed in both mouse and rat collecting ducts (12, 28, 29, 33), NOS3 (endothelial NOS, or eNOS) is also expressed in the IMCD, but at much lower levels than NOS1 (12, 28, 29, 33). NOS1 is also expressed as alternative splice variants; NOS1α is the 155-kDa full-length variant with a PDZ domain, while NOS1β and NOS1γ are NH2-terminal truncated variants without a PDZ domain (4, 7). We recently reported that in the rat IMCD, both NOS1α and NOS1β are expressed, yet in the mouse, NOS1β is predominantly expressed (12, 16). In humans, NOS1α expression has been described for the renal cortex (18), although an evaluation of all the NOS1 splice variants in the human kidney has not been completed.

NOS activity and NO production are regulated by a variety of pathways. For example, NOS undergoes a multitude of posttranslational modifications and/or protein-protein interactions that can either inhibit or stimulate NOS activity and NO production (9). Recently, it has been reported that the protein dynamin-2 (DNM2), interacts with the reductase domain of NOS1 (sequence is identical in all NOS1 variants) and leads to a significant increase in rat collecting duct NO production (15). However, it is unknown whether DNM2 also interacts with NOS1β and what effect this may have on NO production.

In the present study, we first elucidated that NOS1β is predominantly expressed in the human kidney, indicating that the human kidney is more similar to the mouse kidney than the rat kidney with respect to NOS1 expression. We next designed studies to test the hypothesis that DNM2 interacts with NOS1β, leading to an increase in NO production. However, as opposed to our initial hypothesis, the findings indicate that DNM2 acts as a negative regulator of NOS1β. Thus, we further designed studies to test the hypothesis that DNM2 is a negative regulator of collecting duct NOS1β in vivo under high-salt conditions using the novel mouse model with collecting duct-specific NOS1 knockout (CDNOS1KO).

METHODS

**Human kidney samples.** Human kidney lysate samples were purchased from Origene (Rockville, MD). Origene collected these samples from U.S. institutions under strict Institutional Review Board and ethical consent practices. The samples contained 40–80% medulla and were from nontumor, normal renal structures, as determined by a board-certified pathologist. The samples were homogenized by Origene in modified RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40, and 0.25% deoxycholate) plus protease inhibitor cocktail (P2714; Sigma, St. Louis, MO), 0.4 mM Pefabloc SC Plus (Roche 11873601001; Roche, Minneapolis, MN), and PhosStop phosphatase inhibitor (Roche 04906845001). They were then centri-
fuged to remove nonsoluble material and stored at −80°C until purchased. The samples included 3 females and 2 males, of whom there was one African-American, two Caucasians, and 2 patients with unrecorded race. Their ages ranged from 44 to 79 yr. Lysate (10 μg) was separated on an 8% SDS-PAGE gel, as previously described with polyclonal COOH-terminal NOS1-specific antibody (R20, sc-648; Santa Cruz Biotechnology, Santa Cruz, CA) that detects all NOS1 variants (12, 15, 16).

Cell culture. COS7 and mIMCD-3 cells were purchased from American Type Culture Collection (Manassas, VA) and were cultured as previously described (15). Only passages 4−6 were used. All experiments were completed in triplicate and replicated on at least three different days (n = 3 unless noted differently).

Transfection studies. Rat NOS1β in pcDNA 3.1 was purchased from Origene, and the DN2M-GFP or NOS1α constructs used were previously described (15). For communoprecipitation studies, 5 μg of DN2M-GFP construct or empty vector was transfected in 100-mm dishes of confluent cells at a ratio of 1 μg:8 μl of linear polyethylenimine transfection agent (22). The medium was changed after 24 h, and experiments commenced at 48 h posttransfection.

To determine nitrite production, cells were serum-starved for 3 h, washed twice with Hank’s balanced salt solution (HBSS; Mediatech, Manassas, VA), and then incubated in HBSS + 20 U/ml superoxide dismutase + 250 μM L-arginine for 1 h at 37°C at 5% CO2. A subset of cultures was stimulated with 3 μM ionomycin (Sigma) during the hour incubation. HBSS was then snap frozen after analysis for nitrite concentrations by HPLC, as previously described (12, 14, 15). Cells were digested with 20 min of incubation of 0.1 N NaOH, and protein concentrations were determined by Bradford assay (Quickstart, Bio-Rad, Hercules, CA).

Dynamin-2 inhibition and siRNA knockdown. mIMCD-3 cells were grown in 12-well plates and allowed to reach 100% confluency. Cells were then serum starved for 3 h, at which point they were treated for 30 min with various dynamin inhibitors (ab120468; Abcam, Cambridge, MA) [final concentration of 80 μM (15) dissolved in 0.8% DMSO] in HBSS + 20 U/ml superoxide dismutase + 250 μM L-arginine. This panel also includes negative controls. After 30 min, the HBSS was replaced with fresh HBSS + inhibitors or negative controls for an additional 1 h at 37°C at 5% CO2. Afterward, the HBSS was snap-frozen and analysis of nitrite concentrations by HPLC, the cells were digested with 0.1 N NaOH, and protein concentrations were determined by the Bradford assay. Mouse DN2M siRNA (no. SR414809) and scramble control siRNA (no. SR30004) were purchased from Origene. All three DN2M siRNA were combined for maximal inhibition of DN2M. mIMCD-3 cells were siRNA transfected using Lipofectamine RNAiMax (Life Technologies, Carlsbad, CA) using the manufacturer’s reverse transfection protocol. Forty-eight hours after transfection, cell lysates were processed for Western blot analysis to determine knockdown efficiency or nitrite concentration in the cell supernatants. All cells were serum-starved for 3 h before experimentation.

Immunoprecipitation. Western blot, and antibodies. Immunoprecipitations were performed to detect protein-protein interactions between NOS1β and DN2M, and Western blots were performed as previously described (15). IgG controls were performed with mouse or rabbit IgG (Santa Cruz Biotechnology, Dallas, TX) and DN2M/NOS1β lysates passed over the IgG-conjugated beads. Immunogens for human DNMD2 antibody generation were CSFTPQRPVSVHHPGRPPA (residues 760−779) and were generated in rabbits by ProSci (Poway, CA). Immune reactive sera were affinity-purified using antigen cross-linked protein A/G beads. This antigen is 95% homologous to mouse DNMD2. Commercially available antibodies included monoclonal and polyclonal anti-GFP (Santa Cruz Biotechnology, Dallas, TX; sc-9996, sc-8334), polyclonal anti-NOS1 (R20, Santa Cruz, sc-648), monoclonal β-actin (A1978; Sigma).

Identification of dynamin-2 domains. The various domains of mouse dynamin-2 protein (NP_001240822.1) were predicted with the program InterPro (21), and the sequence annotated with the freeware program GeneDoc (Nicholas KB, Nicholas JR, and Deerfield II, DW unpublished data, available at http://genedoc.software.informer.com).

Mouse studies. All animal use and welfare adhered to the NIH Guide for the Care and Use of Laboratory Animals following a protocol reviewed and approved by the Institutional Laboratory Animal Care and Use Committee of the University of Alabama at Birmingham. In house, 3−6-mo-old flox control and CDNS1KO male and female mice were used in the study (12). No statistically significant effects of sex were seen in our study; thus, male and female mice were randomly assigned to the treatment groups. Mice were randomly assigned to either normal-salt (TD.96208, 0.49% NaCl diet, Teklad, Frederick, MD) (Flox mice, n = 24; CDNS1KO mice, n = 18) or high-salt (TD.92034, 4.0% NaCl diet, Teklad) diet for 7 days (Flox mice, n = 38; CDNS1KO mice, n = 24). All mice were euthanized with 50 mg/kg ip of FatalPlus, followed by thoracotomy, and the kidneys were immediately excised.

Fresh IMCD were isolated from two mice (four inner medullas) per preparation, as previously described (12), each preparation representing a single sample (n = 1). IMCD pellets were resuspended in HBSS with final concentrations of 250 μM L-arginine and 20 U/ml superoxide dismutase and +/− vehicle (0.8% DMSO) or 80 μM dynasore for 30 min at 37°C while shaking. After 30 min, the IMCDs were pelleted at 300 g for 5 min, and the HBSS was removed and replaced with fresh HBSS + L-arginine and SOD. After 1 h, the cells were pelleted, and the HBSS and IMCDs were separated, snap frozen, and kept at −80°C until analysis for nitrite and protein concentration, respectively. To determine protein concentration, the IMCD pellet was homogenized in 100 μl of lysis buffer (20 mM Tris, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 μM leupeptin, 2 μM pepstatin A, and 1 mM PMSF, at pH 7.5) with a hand-held disposable pestle system (Fisher Scientific) on ice. The samples were then sonicated with 10 × 1 s bursts on ice. Total protein was determined by Bradford assay. Final sample sizes of independently replicated experiments for each group were as follows: Flox NS: n = 10; Flox HS: n = 10; Flox HS + dynasore: n = 6; CDNS1KO NS: n = 5; HS: n = 4; and HS + dynasore: n = 6.

A communoprecipitation experiment was performed with the control flox IMCD lysates to determine the putative NOS1β-DNM2 interaction in the IMCD. IMCD protein (200 μg) was passed over anti-DNM2-conjugated protein A/G beads or rabbit IgG protein A/G beads, as previously described (15). Western blot analyses were then performed with anti-NOS1 and anti-DNMD2 antibodies, as described above.

In a separate group of mice (n = 4 mice per genotype and diet), the inner medulla from both kidneys was dissected and pooled from flox control and CDNS1KO mice on NS and HS diets, and homogenized in lysis buffer, centrifuged 5,000 × g at 4°C to pellet debris, and used in Western blots to determine DNMD2 expression.

Statistical analyses. For analyses comparing the effect control versus DNMD2 overexpression, siRNA knockdown, or inhibitor, an unpaired, two-tailed Student’s t test was performed. To determine whether there was a significant effect of the dynamin inhibitors mitoTMAB or octoTMAB compared with the negative control on nitrite production or with increasing concentrations of DNMD2 siRNA, a one-way ANOVA was performed with Dunnett’s post hoc test. To test for the effect of transfection and ionomycin stimulation or in the physiological IMCD nitrite experiments for the effect of genotype or diet, two-way ANOVAs were performed with Sidak-Bonferroni post hoc test (α = 0.05).

RESULTS

Human kidney expresses NOS1β. In all 5 human samples of cortex and medulla, we found expression of both NOS1α (155 kDa) and NOS1β (130 kDa) (Fig. 1A). NOS1β was more prominent than the NOS1α, suggesting that NOS1β is the
predominant splice variant expressed in these human renal homogenates. As a positive control for specificity, COS7 cellswere transfected with either NOS1α or NOS1β (Fig. 1B).

**NOS1β and DNM2 interact.** COS7 cells were transfected with NOS1β and DNM2-GFP (Fig. 2, A and B) and as depicted in Fig. 2A, results of the coimmunoprecipitation studies indicated that NOS1β and DNM2 formed a protein-protein interaction. Also, in both empty vector transfected mIMCD-3 cells and mIMCD-3 overexpressing DNM2-GFP (Fig. 2, C and D), NOS1β and DNM2 formed a protein-protein interaction (Fig. 2C). An interaction between both the endogenous DNM2 (100 kDa) and transfected DNM2-GFP (130 kDa) with NOS1β was observed (Fig. 2C).

**DNM2 inhibits NOS1β-mediated nitrite production.** In aqueous solutions, NO is rapidly oxidized to nitrite (19); hence, nitrite levels were measured as an index of NO production. COS7 cells overexpressing NOS1β produced nitrite (Fig. 3A). When DNM2 was also overexpressed with NOS1β in COS7 cells, there was a significant 60% reduction in the nitrite production (Fig. 3A; *P = 0.007). mIMCD-3 cells were also transfected with and without DNM2. Vector-expressing control cellsproduced 421 ± 38 pmol·mg⁻¹·protein⁻¹·h⁻¹ of nitrite and overexpression of DNM2 significantly reduced the mIMCD-3 nitrite production to 224 ± 54 pmol·mg⁻¹·protein⁻¹·h⁻¹ (Fig. 3B; *P = 0.04). Finally, to determine whether DNM2 also inhibits stimulated nitrite production, COS7 cells expressing either NOS1β or NOS1β + DNM2 were acutely stimulated with ionomycin. Ionomycin led to a significant twofold increase in nitrite production by the NOS1β-transfected cells; however, coexpression with DNM2 prevented the ionomycin-dependent increase in nitrite (Fig. 3C, two-way ANOVA: *P\(_{\text{transfection}}\) < 0.001, *P\(_{\text{ionomycin}}\) < 0.001, and *P\(_{\text{interaction}}\) < 0.01).

**DNM2 inhibition increases nitrite production.** Two strategies were employed to inhibit DNM2. First, we used a phar-
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macological approach with a panel of DNM2 inhibitors. These inhibitors targeted different domains of DNM2 (Fig. 4A) and have different chemical scaffolds. Dynamin-2 contains a GTP-Pase, middle, pleckstrin homology (PH), GTPase effector domain, and proline-rich (PRD) domains. The panel of inhibitors targets the GTPase or PH domains. Compared with their respective negative controls, dynasore and dynole (GTPase domain inhibitors) resulted in a significant increase in mIMCD-3 nitrite production (Fig. 4B, P < 0.01). Among the GTPase domain inhibitors used, only iminodin failed to significantly alter mIMCD3 nitrite production (Fig. 4B, P > 0.05). Inhibition of the PH domain (miTAMB or ocTAMB treatment) also resulted in a significant increase in mIMCD-3 nitrite production (Fig. 4C, P = 0.01).

Given that the IMCD also expresses DNM3 (15), siRNA experiments were performed to further confirm the specificity of the inhibition of DNM2. mIMCD-3 cells were transfected with DNM2 or scramble control siRNA, achieving 92 ± 2% knockdown of DNM2 (Fig. 5A). The mIMCD-3 cells with DNM2 knockdown produced significantly more nitrite than scramble control mIMCD-3 cells (Fig. 5B, P < 0.001).

**DNM2 and NOS1β in vivo significance.** Coimmunoprecipitation studies indicated protein-protein interaction between NOS1β and DNM2 in lysates of freshly isolated mouse IMCD (Fig. 6A). Next, flox control mice and mice that lack NOS1 in the CD (CDNOS1KO) (12) provided normal-salt (NS) or high-salt diets (HS) for 7 days, after which IMCDs were isolated. In accord with our previous finding (16), HS diet led to a significant increase in nitrite production in IMCD from flox control mice (NS = 128.3 ± 3.5 pmol·mg protein⁻¹·h⁻¹; HS = 355.7 ± 22.9 pmol·mg protein⁻¹·h⁻¹; P < 0.001). Furthermore, nitrite production by IMCDs from CDNOS1KO mice on the HS diet exceeded that of the NS diet (NS = 91.1 ± 9.4 pmol·mg protein⁻¹·h⁻¹, HS = 187.5 ± 34.0 pmol·mg protein⁻¹·h⁻¹, P < 0.05); however, it was significantly blunted compared with flox control mice fed the HS diet (P_{genotype} < 0.001, P_{diet} < 0.001, P_{interaction} < 0.01). To further elucidate whether DNM2 regulates NO production in vivo, similar experiments utilized acute inhibition of DNM2 with the GTPase inhibitor dynasore. Acute incubation of IMCD with dynasore significantly increased nitrite production by IMCDs from flox control mice fed a HS diet (P_{genotype} < 0.001, P_{treatment} < 0.001, P_{interaction} = 0.02; Fig. 6B). In contrast, although dynasore tended to increase nitrite production by IMCD from CDNOS1KO mice fed a HS diet, this effect did not achieve statistical significance (P > 0.05) and was blunted compared with the response to dynasore in IMCD from flox (Fig. 6B).

Although we previously reported that HS feeding does not increase IMCD NOS expression (16), further studies examined the impact of HS feeding on DNM2 expression. As shown in Fig. 6C, HS feeding resulted in a significant reduction in inner medullary DNM2 expression (P_{genotype} = 0.02, P_{diet} < 0.001, P_{interaction} = 0.089). Flox control mice on a HS diet presented a significantly greater reduction in DNM2 expression compared with NS diet, the HS diet did not significantly reduce inner medullary DNM2 expression compared with NS diet. Although HS feeding did not significantly reduce lower medullary DNM2 expression, Similar studies revealed that CDNOS1KO and flox control mice fed a NS diet displayed similar levels of DNM2 expression. Compared with NS diet, the HS diet did not significantly reduce inner medullary DNM2 expression compared with NS diet; however, DNM2 expression in CDNOS1KO mice on HS diet was significantly greater than that of flox control mice fed the same diet (Fig. 6C).

**DISCUSSION**

In this study, we aimed to test the hypothesis that DNM2 is a positive regulator of NOS1β, as we previously determined that DNM2 increased NOS1β-derived NO production. However, we found that DNM2 was a negative regulator of NOS1β.
such that inhibition or knockdown of DNM2 increased NO production. Moreover, overexpression of DNM2 resulted in a decrease in NO production. Thus, we have identified DNM2 as a novel negative regulator of NOS1β activity.

NOS1 is highly expressed in the IMCD (12, 28, 29, 33), with both NOS1α and NOS1β expressed in the rat, yet mice express predominantly NOS1β in the IMCD (12, 16). In humans, NOS1α has been found in the renal cortex (18); however, this was established using an NH2-terminal antibody that only detects NOS1α. Here, we present evidence, using a COOH-terminal NOS1 antibody that detects all NOS1 variants, that humans express NOS1β. From our five human samples, it appears NOS1β has a greater expression than NOS1α, which suggests that the human kidney is more similar to the mouse kidney than the rat kidney with regard to NOS1 variant expression. Recently, we reported that collecting duct NOS1 is critical for maintaining fluid-electrolyte balance when consuming a HS diet (12). Furthermore, much of the urinary nitrite/nitrate (NOx) is derived from collecting duct NOS1 (12, 31). In both normotensive humans (17) and mice (24, 31), urinary excretion of NOx is increased when fed a high-salt diet. However, urinary NOx is inappropriately low in salt-sensitive hypertensive patients (2, 3, 6, 8, 11), similar to the collecting duct-specific NOS1 knockout mouse that produces inappropriately low urinary NOx and presents with a salt-sensitive blood pressure phenotype (12). Thus, taken together these studies support the utility of the mouse as an appropriate model for humans. Moreover, mouse models are beneficial for testing mechanistic questions about NOS1 activation and regulation of fluid-electrolyte balance and blood pressure control.

Protein-protein interactions lead to regulation of NOS1 activity (34). NOS1α contains a PDZ domain, from which a number of proteins have been determined to interact with NOS1 (34). Recently, our laboratory determined that DNM2 interacts with the reductase domain of NOS1α (15). This novel protein-protein interaction led to a significant increase in NO production. On the contrary, the NOS1β variant lacks the NH2-terminal PDZ domain of NOS1α, but it is otherwise 100% identical in primary sequence with NOS1α. Given that the mouse and human kidney express NOS1β and the reductase domain is conserved in all NOS1 variants, we sought to determine whether DNM2 is also a positive regulator of NOS1β. We confirmed that DNM2 does form a protein-protein interaction with NOS1β; however, using three different approaches, in two different cell lines, we determined that DNM2 inhibits NOS1β-derived NO production. Moreover, increases in intracellular calcium with ionomycin lead to significantly increased NO levels; however, elevated DNM2 levels blocked the calcium-mediated activation of NOS1β. Thus, we have identified a novel NOS1β regulatory pathway.

During high-salt feeding, there are a number of natriuretic and diuretic factors that work to maintain fluid-electrolyte balance. Previously, we demonstrated that collecting duct NOS1 is a modulator in the pressure-natriuresis mechanism and is critical for maintaining fluid-electrolyte balance during HS feeding (12, 13). With a week of HS feeding, the IMCD significantly increases NO production (16), but this increase is significantly blunted in CDNOS1KO mice (Fig. 6). This is also reflected in urinary NO metabolite excretion (NOx), which we previously reported as being significantly blunted in CDNOS1KO mice on a HS diet compared with floc control (12). Thus, IMCD NOS1 and NOS3 contribute to the HS-dependent NO production, although NOS3 is unable to fully compensate for the loss of CD NOS1. NOS2 is not normally expressed in the mouse inner medulla (12). To determine whether DNM2 influences NOS1 and NOS3 activity in vivo, we placed flox control and CDNOS1KO mice on a HS diet for 7 days. To further determine whether DNM2 activity as it relates to NOS1-dependent NO production is altered with HS feeding, we acutely inhibited DNM2 activity in IMCDs from flox and CDNOS1KO mice on HS diets with dynasore (20). Acute DNM2 inhibition in isolated IMCDs from mice on a HS diet resulted in a statistically significant increase in NO production in flox mice but not CDNOS1KO mice. However, there was a tendency for DNM2 inhibition to increase IMCD NO production in the CDNOS1KO mouse, suggesting a small contribution of NOS3. This finding is in contrast to the published report that used bovine aortic endothelial cells and purified DNM2 to demonstrate that DNM2 activates NOS3 (5). Our current findings suggest that, in the IMCD, DNM2 acts as an inhibitor of NO production and a negative regulator of NOS1β and, most likely, NOS3. We determined that an HS diet leads to a decrease in inner medullary DNM2 expression in flox control animals, but not in the inner medulla from CDNOS1KO mice. This observation suggests that DNM2 expression is regulated by NOS1 activity during HS feeding, although the mechanism remains to be determined. Taken together, these data suggest that inner medullary DNM2 ex-
pression is reduced with HS feeding, and the residual DNM2 activity acts as a break to regulate NO production. We speculate that this process is critical to fine-tune and balance NO production, to match the natriuretic and redox states of the nephron, and to ensure that the excess salt is excreted, while preventing aberrant NO production. Excess NO leads to nitrative stress, including the formation of peroxynitrite, which can lead to DNA damage, protein nitration, fatty acid nitration, and ultimately cytotoxicity (26, 32). In the endothelium, alterations in DNM2 activity result in increased permeability under hypoxic conditions, which is mediated by a peroxynitrite-dependent mechanism (25).

Dynamin-2 plays a critical role in a variety of cellular processes. The classic function is to regulate endocytosis of plasma membrane proteins, via its scission of budding vesicles (for review, see Ref. 10), although studies suggest DNM2 also regulates Golgi network vesicles, trafficking, and fusion processes (10). Moreover, a novel role of DNM2 regulation of the release of apical vesicular carriers in the apical recycling endosome pathway was recently elucidated (30). Thus, it is evident that DNM2 functions in more than just scission of vesicles from the plasma membrane. Dynamin isoforms contain a PRD that interacts with a variety of proteins. For example, proteins that contain an SH3 domain interact with the DNM2 PRD and result in an increased DNM2 GTPase activity (23). Interestingly, DNM2 also increases enzymatic activity of its binding partner. Previous studies determined that the reductase domain of NOS3 (endothelial NOS) interacts with the PRD domain of DNM2, resulting in an increased NOS3 activity (5). Furthermore, we reported that the NOS1 reductase domain interacts with DNM2, stimulating NOS1 activity (15). Inhibition of the DNM2 GTPase domain with dynasore resulted in reduced NO production by NOS1 (15). In the current study, we demonstrated that DNM2 interacts with NOS1β and likely NOS3 to inhibit IMCD NO production. Inhibition of either the GTPase or PH domains by a variety of
inhibitors led to a stimulation of NOS1 activity. Knockdown of endogenous DNM2 also led to a stimulation of mouse IMCD NO production. From these findings, we conclude that the GTPase and PH domains of DNM2 are involved in NO production by the IMCD.

The mechanism(s) of DNM2 activation of NOS1α and inhibition of NOS1β remain speculative. Cao et al. (5) determined that the PRD domain of DNM2 interacts with the reductase domain of NOS3, resulting in NOS3 activation due to an increase in electron transfer between the FAD and FMN domains of NOS3. We previously determined that the reductase domain of NOS1 (which is 100% conserved between NOS1α and NOS1β) interacts with DNM2; however, the DNM2 domain involved in this interaction remains to be determined. From the current study with NOS1β, it is the GTPase and PH domains of DNM2 that inhibit NO production; thus, we would propose that a reduction in electron transfer occurs. Other possibilities include changes in substrate affinity, including a reduction in calcium/calmodulin binding, BH4, or perhaps even L-arginine. Subcellular localization, posttranslational modifications, and protein-protein interactions are all critical for NOS activity (1). It is well established that NOS3 undergoes multiple posttranslational modifications that can either increase or decrease activity. On the contrary, NOS1 regulation is regulated through protein-protein interactions. Although, purified NOS1β has ~80% of the activity of NOS1α (4), we know very little about the regulatory pathways of NOS1β. For example, Ser/Thr phosphorylation sites are distinct between NOS3 and NOS1 (1) and may be further distinct between NOS1α and NOS1β. Furthermore, there may be unique protein-protein interactions among DNM2, the NOS isoforms, and splice variants, and other yet to be determined proteins that confer distinct regulation of NOS activity. These findings clearly demonstrate a need for further investigation into the molecular mechanisms involved in the distinct patterns of DNM2 regulation of the NOS isoforms and splice variants.

Perspectives and Significance

In summary, the renal medulla expresses NOS1 splice variants, with mice and human medullas expressing greater levels of the NOS1β variant, as well as rats expressing both NOS1α and NOS1β (16, 27). NOS1α and NOS1β form protein-protein interactions with DNM2 resulting in activation of NOS1α but inhibition of NOS1β. Thus, DNM2 is a novel NOS1-interacting protein that can either activate or inhibit NOS1 depending on the splice variant that is involved in the association. This complexity may be important for regulation of NO production in response to different stimuli. We previously reported that mice and rats regulate medullary NO production in distinct manners; in response to a HS diet, rats increase expression of NOS1β over time, while mice maintain a constant level of NOS1β expression (16). HS feeding leads to a significant increase in mouse IMCD NO production (16), and here, we present evidence that although DNM2 expression is reduced with HS feeding, there is residual DNM2 activity that limits the increase in NO production. We speculate that this may regulate the redox state of the IMCD and prevent the production of toxic levels of NO. In renal diseases, increases in nitrosylation and oxidative stress result in significant renal damage (26, 32). Alternatively, salt-sensitive hypertension is associated with renal NO deficiency (3, 11). Thus, we have identified DNM2 as a new target that warrants further investigation to determine whether deranged DNM2 regulation is causative in renal diseases.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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