Dynamin activates NO production in rat renal inner medullary collecting ducts via protein-protein interaction with NOS1

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Dynamin activates NO production in rat renal inner medullary collecting ducts via protein-protein interaction with NOS1. Am J Physiol Renal Physiol 301: F118–F124, 2011. First published April 13, 2011; doi:10.1152/ajprenal.00534.2010.—We hypothesized that nitric oxide synthase (NOS) isoforms may be regulated by dynamin (DNM) in the inner medullary collecting duct (IMCD). The aims of this study were to determine which DNM isoforms (DNM1, DNM2, DNM3) are expressed in renal IMCDs, whether DNM interacts with NOS, whether a high-salt diet alters the interaction of DNM and NOS, and whether DNM activates NO production. DNM2 and DNM3 are highly expressed in the rat IMCD, while DNM1 is localized outside of the IMCD. We found that DNM1 interacts with NOS1α, NOS1β, and NOS3 in the inner medulla of male Sprague-Dawley rats on a 0.4% salt diet. DNM2 interacts with NOS1α, while DNM3 interacts with both NOS1α and NOS1β. DNM2 and DNM3 do not interact with NOS3 in the rat inner medulla. We did not observe any change in the DNM/NOS interactions with rats on a 4% salt diet after 7 days. Furthermore, NOS1α interacts with DNM2 in mIMCD3 and COS7 cells transfected with NOS1α and DNM2-GFP constructs and the NOS1 reductase domain is necessary for the interaction. Finally, COS7 cells expressing NOS1α or NOS1α/DNM2-GFP had significantly higher nitrite production compared with DNM2-GFP only. Nitrite production was blocked by the DNM inhibitor dynasore or the dominant negative DNM2K44A. Ionomycin stimulation further increased nitrite production in the NOS1α/DNM2-GFP cells compared with NOS1α only. In conclusion, DNM and NOS1 interact in the rat renal IMCD and this interaction leads to increased NO production, which may influence NO production in the renal medulla.

NITRIC OXIDE (NO) is a critical regulator of several physiological pathways in the kidney. Mattson’s laboratory previously demonstrated that the highest NOS activity was in the inner medullary collecting duct (IMCD) in the rat (37). All NOS synthase (NOS) isoforms are expressed in various segments of the rat nephron (24, 25, 37), specifically NOS1 (neuronal NOS) is expressed in the macula densa, cortical and medullary collecting duct, thick ascending limb, and vasa recta (3, 18, 37); NOS2 (inducible NOS) is constitutively expressed in the thick ascending limb of the nephron (12, 19); and NOS3 is expressed in endothelial cells throughout the kidney, thick ascending limb, and collecting duct cells (3, 33). The NOS1 gene has many alternative promoters (4, 10) and reports showed that there are three NOS1 splice variants expressed in the rat nephron termed NOS1α, NOS1β, and NOS1γ (17, 22, 28). The reductase domain of the three NOS isoforms and the NOS1 splice variants are all highly conserved (1, 39).

Protein–protein interactions and subcellular localization are important regulators of NOS activity and consequently NO production. Several proteins have been shown to interact with NOS and regulate NO production (1, 39). We previously reported that NOS activity within the renal inner medulla is over fourfold higher in the intracellular membrane fraction relative to the cytosolic and plasma membrane fractions (31). Of the known NOS-interacting proteins that activate NOS and may be relevant in various subcellular compartments (23), dynamin (DNM) would be considered one of the top candidate proteins.

DNMs belong to the large GTPase family of proteins and are comprised of several domain structures that facilitate interactions, specifically a lipid-binding pleckstrin homology domain, a GTPase effector domain, and a proline-rich COOH-terminal domain (e.g., 13). DNMs are essential in the endocytotic pathway, whereby these proteins are involved in the scission of invaginated clathrin-coated vesicles (35) and caveolae (38). There are three members that are differentially expressed; dynamin-1 (DNM1) is predominately expressed in the nervous system (21), while dynamin-2 (DNM2) and dynamin-3 (DNM3) are ubiquitously expressed (9, 16, 29). Additionally, up to 25 splice variants have been described (5). Cao et al. (7) previously demonstrated that DNM/NOS3 interact in bovine aortic endothelial cells and determined that the dynamin-rich COOH-terminal domain of DNM2 directly interacts with the FAD region of the reductase domain of NOS3. This direct interaction was demonstrated to increase NOS3 activity (7). Wang et al. (36) further showed that DNM1 interacts with NOS3 in HEK 293 cells, although it is unclear whether DNM1 also activates NOS3 in these cells.

Little is known about whether NOS1 isoform interacts with DNM, and specifically whether these proteins interact in the kidney. Given the high degree of homology between the reductase domains of NOS1 and NOS3, and the high NOS activity in the rat renal inner medulla and IMCD (37), we hypothesized that DNM interacts with NOS1 and/or NOS3 in the renal inner medulla and regulates NO production. The initial aims of this study were to determine the expression of DNM isoforms in the renal inner medulla and whether DNM and NOS interact in renal inner medullary tissue. These studies revealed that indeed DNM and NOS interact in the renal tissue, thus we further elucidated whether the interaction is influenced by a high-salt diet and/or whether the DNM/NOS1 interaction activates NO production.

EXPERIMENTAL DESIGN AND METHODS

Animals. All animal protocols were in accordance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Utilization Committee at the Medical College of Georgia. Male Sprague-Dawley rats (230–250 g) were purchased...
from Harlan Labs and maintained at the lab animal services facility. Animals were fed ad libitum a 0.4% NaCl (normal salt, Harlan Teklad, Indianapolis, IN) diet with free access to tap water. After acclimation to the animal facility (7 days), rats were maintained on 0.4% NaCl diet (normal salt) or given 4.0% NaCl (high salt, Harlan Teklad) diet for 7 days. Rats were anesthetized with pentobarbital sodium (Sigma, St. Louis, MO) and killed by thoracotomy. The left and right kidneys were immediately excised, decapsulated, and the inner medulla was dissected and snap-frozen in liquid nitrogen. All tissues were kept at −80°C until analysis. For immunohistochemical analysis, rats were anesthetized as described and the kidneys were perfusion-fixed with 10% neutral-buffered formalin (NBF; Fisher Scientific, Pittsburgh, PA) for 10 min. The kidneys were excised, decapsulated, sectioned sagitally into thirds, and further fixed in 10% NBF for 24 h at room temperature. The kidneys were then embedded in paraffin and sectioned.

**Immunohistochemistry.** Five kidney sections, of 5-μm thickness, per animal were analyzed as previously described (30). The tissue sections were incubated overnight at 4°C with the isoform-specific antibodies to DNM1–3 (1:2,000; Abcam, Cambridge, MA). The secondary antibody was goat anti-rabbit, horseradish peroxidase polymer (Biocare, Concord, CA) incubated for 20 min at room temperature in a humidified chamber. Immunoreactivity was detected by diaminobenzidine (Dako, Carpinteria, CA). Slides were visualized with the Olympus BX40 microscope, affixed with a DP70 Camera.

**IMCD3 and COS7 transfections.** Mouse IMCD-3 (mIMCD3) and COS7 cells were purchased from ATCC (Manassas, VA). One day before transfection, 2 × 10⁵ cells were plated in growth medium (DMEM or DMEM:F-12, respectively) containing 5% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) resulting in cells 90–95% confluent at time of transfection in 100-mm plates. Fugene 6 transfection agent (Roche, Indianapolis, IN) was added to serum- and antibiotic-free media at a concentration of 1 μl/g in 6 μl of Fugene and incubated for 15 min at room temperature. The fugene/DNA media was added to each plate and gently rocked back and forth. The cells were incubated at 37°C in a 5% CO₂ incubator for 48 h with the medium changed after 6 and 24 h of transfection. All transfection combinations were repeated four to five independent times.

**Immunoprecipitation protocol.** Cerebellar or inner medullary tissues were homogenized in 500 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, 1 mM phenylmethylsulfonyl fluoride, pH 7.5) centrifuged at 4,000 g. Protein concentration of the supernatant determined with the Bio-Rad BCA kit (Carlsbad, CA). Protein (750 μg) was incubated with Protein A/G beads conjugated with pan-dynamin (Upstate Millipore, Billerica, MA) or dynamin isoform-specific antibodies (10 μg antibody/immunoprecipitate; Abcam). Transfected cells were harvested, washed twice in 10 mM PBS (Invitrogen), and lysed in lysis buffer. Cell lysates were centrifuged at 4,000 g for 10 min at 4°C. The supernatant was retained and protein concentration was determined (BCA assay, Bio-Rad). Three micrograms of green fluorescent protein (GFP) antibody or 10 μg of NOS1-specific antibody was incubated with 50 μl of preblocked protein A/G agarose bead slurry (Santa Cruz Biotechnologies, Santa Cruz, CA) for 1 h at 4°C. Excess antibody from the immunoprecipitate complex was removed by washing the beads 3× with lysis buffer. Next, 500 μg of protein were mixed with 50 μl antibody-conjugated beads in an empty Bio-spin disposable column (Bio-Rad) overnight at 4°C. The beads were then washed 3× with lysis buffer + protease inhibitors. Proteins were eluted by boiling the beads in 2× Laemmli buffer (Bio-Rad) with 5% beta-mercaptoethanol for 5 min at 90°C. Immunoprecipitated proteins were run on 8% SDS-PAGE, blotted to PVDF membranes, and detected with anti-GFP (1:10,000; Clontech, Mountainview, CA), monoclonal, anti-COOH-terminus NOS1 (1:1,000; BD Scientific, San Jose, CA), or polyclonal anti-NOS3 (1:100; Santa Cruz Biotechnologies) antibodies and visualized with goat anti-mouse secondary antibody (Invitrogen) or goat anti-rabbit secondary antibody (Rockland, Gilbertsville, PA) using the Odyssey Infrared Imaging System (Licor Biosciences).

**Cellular fractionation of rat IMCDs.** Rat IMCDs were freshly perfusion-fixed with 10% neutral-buffered formalin (NBF; Fisher Scientific, Pittsburgh, PA) for 24 h at room temperature. The kidneys were then embedded in paraffin and sectioned.

**Statistical analysis.** For nitrite determination, data were plotted as means ± SE. To test the significance of overexpressing NOS1 and DNMD2-GFP in COS7 cells, anova was performed using the Student’s t-test as the post hoc test. To test the significance of ionomycin on nitrite production in these cells, a two-way ANOVA with unpaired Student’s t-test was performed with an α = 0.05 considered significant. These results were obtained from 2–3 independent cell preparations. Each data point represents the mean ± SE of 3–5 experiments per group.
within the inner medulla localized DNM1 in cells surrounding the collecting ducts and with low expression localized in the collecting ducts (Fig. 1, D and G). DNM2 (Fig. 1, E and H) and DNM3 (Fig. 1, F and I) appeared to be exclusively expressed in the collecting duct with other structures demonstrating very low or no immunoreactivity. Finally, DNM isoforms, NOS1, and NOS3 subcellular localization was determined in isolated rat IMCD. As seen in Fig. 1J, NOS1, DNM2, and DNM3 are expressed in the plasma membrane, intracellular membrane, and soluble fractions, while NOS3 was expressed in the plasma membrane and intracellular membrane fractions only. DNM1 was expressed at very low levels in the rat IMCD subcellular fractions.

**DNM/NOS interactions in vivo.** The rat cerebellum expresses high levels of NOS1α and DNM so initially we probed for NOS1/DNM interactions in the cerebellum using a pan-DNM antibody and found that NOS1 interacts with DNM in the cerebellum (Fig. 2A).

Lysates from inner medulla of the rat kidney were immunoprecipitated with DNM isoform-specific antibodies and immunoblotted with a COOH terminus NOS1-specific antibody to determine which DNsMs may interact with NOS1α and/or NOS1β. As observed in Fig. 2, C and E, DNM1 and DNM3 interact with NOS1α and NOS1β (n = 6–7), while DNM2 interacts with NOS1α (Fig. 2D; n = 6). The DNM/NOS1 interactions were similar in tissues from rats on normal- and high-salt diets (Fig. 2B–E; n = 6–7).

Similar to a previous report in endothelial cells (36), we found that DNM1 and NOS3 interact in the rat renal inner medulla (Fig. 2B; n = 6). However, DNM2 and DNM3 do not appear to interact with NOS3 in the rat renal inner medulla (data not shown; n = 10). The DNM1/NOS3 interaction was similar in tissues from rats on a normal-salt and high-salt diet (Fig. 2B; n = 7).

**DNM2/NOS1 interaction in vitro.** mIMCD3 and COS7 cultured cells were transfected with NOS1α and DNM2-GFP constructs (Figs. 3 and 4), mIMCD3 cells express NOS1β endogenously (Fig. 3A), whereas COS7 cells do not express NOS isoforms. We confirmed that DNM2 and NOS1 interact in these in vitro settings (Figs. 3C and 4C; n = 4). In addition, we found an interaction between endogenous NOS1β expressed in mIMCD3 cells and DNM2-GFP (Fig. 3C; n = 4). In addition, COS7 cells were transfected with the NOS1 reductase domain (identical domain in NOS1α and NOS1β) and/or DNM2-GFP and immunoprecipitated with a COOH terminus NOS1-specific antibody. We determined that DNM2 interacts with the reductase domain of NOS1 (Fig. 5).

**DNM2/NOS1 interaction increases nitrite production.** COS7 cells were transfected with DNM2-GFP, NOS1, or NOS1 and DNM2-GFP to determine nitrite production as an index of NOS1 activity. Nitrite production was below detection in the COS7 cells transfected with DNM2-GFP alone (Fig. 6A). Nitrite production significantly increased in cells transfected with NOS1α (193 ± 20 pmol·mg protein⁻¹·h⁻¹; n = 6) and further increased in the NOS1α + DNM2-transfected cells (390 ± 65 pmol·mg protein⁻¹·h⁻¹; n = 6; P < 0.001; Fig. 6A). Stimulation with ionomycin increased nitrite production in both the NOS1α (574 ± 34 pmol·mg protein⁻¹·h⁻¹; n = 5) and NOS1 + DNM2-transfected cells (2,122 ± 592 pmol·mg protein⁻¹·h⁻¹; n = 4; Fig. 6B). Basal and ionomycin-stimulated nitrite production was inhibited by incubation with dynasore for 1 h (Fig. 7A; n = 3, ionomycin P < 0.0001, dynasore...
We confirmed that incubation with dynasore was not cytotoxic (Supplemental Fig. S2). Additionally, the DNM2-specific contribution to nitrite production by mIMCD3 cells was inhibited by the dominant negative DNM2-K44A as well as the ionomycin-stimulated increase in nitrite (Fig. 7B; n = 3–5, ionomycin P = 0.015, transfection P = 0.48, interaction P = 0.42). Finally, nitrite production was completely abolished by incubation of freshly isolated rat IMCDs with 80 μM dynasore (Fig. 7C; n = 4).

**DISCUSSION**

The major findings of this study show that NOS1 interacts with DNM isoforms in rat cerebellar and renal inner medullary tissues as well as the renal IMCD. We also found that NOS3 interacts with DNM1 in the renal inner medulla. We found that DNM2 and DNM3 are highly expressed within the IMCD, while DNM1 has a relative low level of expression in the CD.

Furthermore, we demonstrated that DNM2 activates NOS1 and stimulates NO production with a specific interaction of DNM2 with the reductase domain of NOS1. Moreover, inhibition of DNM blocked NO production in rat renal IMCDs.

DNMs play an integral role in the endocytotic pathway (13) as well as being involved in the regulation of protein functions through protein-protein interactions (34). DNMs contain proline-rich and pleckstrin homology domains that interact with numerous proteins and subcellular components including tu-

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Although we only observed NOS1 and DNM3 are expressed in the same subcellular compartments of IMCDs. We observed that NOS1, DNM2, and DNM3 are also expressed in all three subcellular fractions: cytosolic or soluble fraction, while NOS3 was only found in the plasma membrane, intracellular membrane fraction, and cytosolic fraction, leading to an increase in NO production. Cao et al. (6) reported that the DNM2/NOS3 interaction is more sensitive to intracellular Ca$^{2+}$ signaling within the IMCD. In the conditions of our study, NOS3 only interacts with DNM1 in the renal tissue lysates. Since DNM1 is expressed at very low levels in the IMCD, we concluded that DNM-dependent regulation of NOS in the IMCD is predominately with the NOS1 variants over NOS3. It is unclear whether the DNM/NOS1 interactions result in translocation among different subcellular compartments. Within the vascular endothelium, DNM2 is proposed to regulate NOS3 through direct activation of the enzyme via stimulation of the reductase domain (7), but also through facilitating trafficking and translocation (6, 8). The dominant negative DNM2 (K44A) anchors NOS3 to the plasma membrane and in various endothelial cell lines shown to inhibit NO production (8, 26). The mechanism of inhibition by DNM2 K44A is through an effect on NOS3 trafficking and not due to the mutation itself; the K44A mutation is distant from the region that directly binds to NOS3 (8, 26). We propose that a similar mechanism may occur between DNM2 and/or DNM3 with NOS1 within the IMCD. Future studies will determine whether the interaction of the DNM1 and NOS1 splice variants is regulated differentially among the subcellular compartments.

Renal medullary NOS1-dependent NO is a mediator of natriuresis and diuresis (20, 25). These new findings indicate that DNM regulates NOS1-derived NO production in the IMCD, thus validating the physiological relevance of the DNM/NOS1 interaction. We found that the DNM/NOS interactions in the inner medullary tissue of rats fed a normal- and a high-salt diet were similar. Previously, we reported that NOS expression and activity were the highest in the intracellular membrane fraction in the renal inner medulla under both normal-salt and high-salt diet conditions (31). Taken together, these observations suggest that under high-salt conditions, NO production in the renal inner medulla may not be regulated by differential NOS subcellular compartmentalization or interaction with DNM. Thus, we reasoned that high-salt conditions may stimulate intracellular Ca$^{2+}$ signaling within the IMCD leading to an increase in NO production. Cao et al. (6) reported that the DNM2/NOS3 interaction is more sensitive to intracellular Ca$^{2+}$ signaling in endothelial NO production. Therefore, we determined whether the DNM2/NOS1 interaction is sensitive to increases in intracellular Ca$^{2+}$. Indeed, we demonstrated that the DNM2/NOS1 interaction is sensitive to increases in intracellular Ca$^{2+}$. Indeed, we demonstrated...
that ionomycin led to a greater than threefold increase in nitrite production in the NOS1/H9251/H11001 DNM2-transfected cells, while only a twofold increase in nitrite production in the NOS1/H9251-transfected cells, thus indicating that an increase in intracellular Ca\textsuperscript{2+} significantly enhances NO production when DNM2 is bound to NOS1 as opposed to NOS1 in the absence of DNM2. Clearly, more research is needed to understand how DNM may regulate NO production in the IMCD during a high-salt intake.

In conclusion, DNM regulates NO production in rat IMCD via a protein-protein interaction with NOS1. Future studies will elucidate whether this interaction results in an increase in NOS trafficking and NO production in subcellular compartments necessary to regulate sodium excretion.

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DYNAMIN AND NOS1 IN THE COLLECTING DUCT

REFERENCES


