Novel role of AQP-1 in NO-dependent vasorelaxation

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Nitric oxide (NO) produced by endothelial cells diffuses to vascular smooth muscle cells to cause dilatation of the renal vasculature and other vessels. Although it is generally assumed that NO moves from cell to cell by free diffusion, we recently showed that aquaporin-1 (AQP-1) transports NO across cell membranes. AQP-1 is expressed in endothelial and vascular smooth muscle cells. We hypothesized that diffusion of NO into vascular smooth muscle cells and out of endothelial cells is facilitated by AQP-1, and transport of NO by AQP-1 is involved in endothelium-dependent relaxation. In intact aortic rings from AQP-1−/− mice, vasorelaxation induced by acetylcholine (which increases endogenous NO) was reduced (P < 0.0001 vs. control). No differences were found in the relaxation caused by NOS3 expression was 13.4 pmol NO/mg for AQP-1−/− mice, 188% increase (P < 0.01). We conclude that 1) AQP-1 facilitates NO influx into vascular smooth muscle cells, 2) AQP-1 facilitates NO diffusion out of endothelial cells, and 3) transport of NO by AQP-1 is required for full expression of endothelium-dependent relaxation.

MATERIALS AND METHODS

Materials. Cell culture reagents were all from GibCO-Invitrogen (Carlsbad, CA). Unless otherwise specified, all other reagents were from Sigma (St. Louis, MO).

Animals. Breeding pairs of AQP-1−/− mice were a gift from Dr. H. Brooks at the University of Arizona (Tucson, AZ). They were bred in the animal facility of Henry Ford Hospital by the Transgenic Mouse Core of the Division of Hypertension and Vascular Research. Their respective controls, CD1 mice (37), were obtained from Charles River (Kalamazoo, MI). C57Bl/6J mice were obtained from Jackson (Bar Harbor, ME). Only animals weighting 24–28 g on the day of the experiment were used. All protocols were carried out in accord with the guidelines of the Institutional Animal Care and Use Committee.

Aortic relaxation in response to acetylcholine, spermine NONOate, sodium nitroprusside, and phosphodiesterase 5 inhibition. Male AQP-1−/− and CD1 control mice were anesthetized with ketamine (100 mg/kg body wt ip) and xylazine (20 mg/kg body wt ip). Thoracic aortas were removed, cleared of adhering connective tissue, and cut into rings 2 mm in length. When necessary, the endothelium was removed by passing a piece of 5-cm 4.0 surgical suture through the lumen of the vessel six times. Aortic rings were mounted in a vessel myograph (Multi Myograph System-610M, Danish Myo Technology, Aarhus, Denmark) and bathed with buffer containing (in mM): 120 NaCl, 25 NaHCO3, 4.7 KCl, 1.18 KH2PO4, 1.18 MgSO4, 2.5 CaCl2, 0.026 ethylenediaminetetraacetic acid (EDTA) disodium salt, and 5.5

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glucose, pH 7.4, at 37°C gassed with 95% O2-5% CO2. Resting force was set at 6.9 mN (700 mg). Active force was recorded isometrically.

Preparations were allowed to equilibrate for 1 h, replacing the buffer at 20-min intervals. The rings were constricted twice with 100 mM KCl (using the above buffer, except that 100 mM KCl was achieved by equimolar substitution of NaCl). After removal of KCl, preparations were allowed to reach equilibrium for 40 min, replacing the buffer at 20-min intervals. When the NOS inhibitor Nω-nitro-l-arginine methyl ester (l-NAME; 1 mM) or the superoxide scavenger (Tempol, 100 μM) was used, these drugs were added to the bath 20 min before phenylephrine. The vessels were constricted by adding a concentration of phenylephrine that caused 80% constriction. When a plateau was reached, we obtained concentration-response curves for relaxation induced by either acetylcholine (to stimulate NO production by endothelial cells), spermine NONOate (an NO donor that spontaneously releases NO into the bath; Cayman Chemicals, Ann Arbor, MI), sodium nitroprusside (an NO donor that releases NO intracellularly), or the PDE5 inhibitor 4-[(3′,4′-methyleneedioxy)benzyl]amino]-6-methoxyquinazoline (that increases endogenous cGMP levels; Calbiochem, San Diego, CA). Complete endothium removal was tested by adding 1 μmol/l acetylcholine when appropriate. The degree of relaxation was calculated as a percentage of PE tension.

**Primary cultures of mouse endothelial and vascular smooth muscle cells.** Endothelial and vascular smooth muscle cells from mouse thoracic aortas were isolated according to the methods of Kobayashi et al. (34) and Ray et al. (53) with some modifications. Briefly, mice were anesthetized with xylazine (20 mg/kg body wt) and ketamine (100 mg/kg body wt). The thorax was opened and the abdominal aorta was cut at the middle and perfused from the left ventricle with 1 ml PBS (140 mM NaCl, 10 mM phosphate buffer, and 3 mM KCl, pH 7.4) containing 1,000 U/ml heparin. The thoracic aorta was then dissected, and fat and connective tissue were removed from both ends. Five-centimeter PE-50 tubing was inserted into the proximal portion of the aorta. The inside of the lumen was washed with serum-free medium, using a 3-ml syringe and a sterile 25-gauge needle. The distal end of the aorta was closed with 6.0 surgical suture and the lumen filled with collagenase type 2 solution (Worthington, Lakewood, NJ, 2 mg/ml dissolved in serum-free DMEM). The outside of the vessel was rinsed by passing it through 0.5 ml medium three times and transferred to a clean plastic dish containing serum-free medium without collagenase. After incubation for 30 min at 37°C, endothelial cells were removed from the aorta by flushing it with 5 ml DMEM containing 20% heat-inactivated FBS. Cells were collected by centrifugation at 300 g for 5 min, resuspended with 2 ml 20% FBS-DMEM, and cultured in 35-mm collagen type I-coated inserts (0.4 μm, 4.7-cm² area, Corning Costar, Cambridge, MA). After 2 h, medium was replaced by DMEM plus 20% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 1× nonessential amino acids (GIBCO cat. no. 11140-050), 1 mM sodium pyruvate, 100 μg/ml heparin, and 100 μg/ml endothelial cell growth supplements. Two mice were necessary to obtain a single plate of confluent endothelial cells after 1 wk of incubation.

After endothelial cells were removed, the adventitia was removed. The aorta was cut into 1- to 2-mm pieces, transferred to a small tissue culture tube containing 1 ml collagenase (1.3 mg/ml) in serum-free DMEM, and digested at 37°C for 3.5 h in an incubator with 5% CO2. Tissue was disturbed every 30 min by pipetting up and down. Cells were collected by centrifugation at 300 g for 5 min at room temperature and rinsed with 5 ml DMEM high glucose supplemented with 10% bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Then, they were resuspended, placed in two wells of a 24-well plate containing a round glass coverslip 13 mm in diameter, and used 5 days later.

To make sure our primary cultures were not contaminated with other cell types, endothelial and vascular smooth muscle cells isolated from CD1 aortas were plated on glass coverslips until confluency. For endothelial cells, laminin- and fibronectin-treated coverslips were used. Cells were fixed in ice-cold 4% paraformaldehyde for 30 min. After incubation for 30 min in 5% albumin/Tris-buffered saline (TBS; 20 mM tris base, 137 mM NaCl, pH 7.6) 0.1% Tween, endothelial cells were incubated with a 1:200 dilution of a NOS3 monoclonal antibody (BD Biosciences, San Diego, CA) and vascular smooth muscle cells with 1:200 of monoclonal vascular smooth muscle cells α-actin antibody (Dako, Carpenteria, CA). Cells were washed twice for 5 min with TBS-T, incubated for 30 min with 1:500 Alexa Fluor 488 goat anti-mouse antibody (Molecular Probes-Invitrogen, Carlsbad, CA), then rewashed twice for 5 min with TBS-T, and mounted using Fluormount-G (Southern Biotech, Birmingham, AL). Cells were visualized under a ×100 oil objective and the secondary antibody was excited with an argon/krypton laser at 488 nm. Emitted fluorescence was visualized with a scanning laser confocal microscope (Naror Instruments, Middleton, WI). Primary cultures of endothelial cells were 98% pure. Primary cultures of vascular smooth muscle cells were 96% pure.

**Western blot.** Western blotting was performed as routinely done in our laboratory (23, 26). For detection of AQP-1, 15 μg endothelial cells and 5 μg vascular smooth muscle homogenates were loaded onto the same 12% polyacrylamide gel, electrophoresed, and transferred. Membranes were incubated for 1 h with 1:1,000 dilution of AQP-1 polyclonal antibody (Alpha Diagnostics, San Antonio, TX). To make sure the AQP-1 antibody was specific, we used purified AQP-1 as a positive control for our Western blots. We observed the expected molecular weight bands for AQP-1 by either Silver stain or Western blot. Additionally, we used two different antibodies raised against different epitopes of AQP-1 (Alpha Diagnostics and BD Biosciences), and both antibodies gave us the expected molecular weight bands for AQP-1 by Western blot. For detection of vascular smooth muscle cell α-actin, 1:200 of monoclonal antibody was used (Dako). For detection of NOS3, 2 μg endothelial cells homogenates were loaded onto a 8% polyacrylamide gel and a 1:1,000 dilution of a NOS3 monoclonal antibody was used (BD Biosciences). Membranes were incubated for 1 h using a 1:1,000 dilution of the appropriate secondary antibody. Quantification of the bands was performed by densitometry. When a different amount of protein was loaded, densitometric values were corrected accordingly and expressed as O.D. units per 10 μg of protein.

**Measurements of NO influx.** Primary cultures of vascular smooth muscle cells were grown on glass coverslips until 60% confluence and placed in a temperature-regulated chamber at 37°C. The flow rate of the bath was 0.4 ml/min [HEPES-buffered physiological saline: (in mM) 130 NaCl, 2.5 NaH2PO4, 4 KCl, 1.2 MgSO4, 6 alanine, 1 Na3 citrate, 5.5 glucose, 2 Ca (lactate)2, 10 HEPES at pH 7.4]. Cells were loaded by adding 4 μM DAF-2 DA to the medium for 30 min and then washed with physiological saline for 30 min. The dye was excited at 488 nm with an argon/krypton laser, and fluorescence was emitted by NO-bound dye was measured with a scanning laser confocal microscope (Noror Instruments). After stable baseline fluorescence was established, the NO donor spermine NONOate [500 μM, which releases 5 μM NO as measured by a precalibrated NO-selective sensor (24)] was added to the bath. The increase in fluorescence, representing the increase in intracellular NO, was measured once every 5 s for 50 s. Rates of NO influx were calculated from the slope of the initial rates of increases in fluorescence. Results were expressed in fluorescence units/second.

**Measurements of NO release.** NO release was measured as routinely done in our laboratory (22, 24). Briefly, confluent primary cultures of endothelial cells were transferred to a temperature-regulated system and maintained at 37°C. The atmosphere contained 95% O2-5% CO2. A precalibrated NO selective sensor (amiNO-Flat, Harvard Apparatus, Holliston, MA) was placed on top of the cells. Once a baseline was obtained, 1 μM acetylcholine and 250 μM L-arginine (the substrate for NOS) were added to the apical side of the cells and NO release was measured continuously for 5 min (26). At the end of the experiment, cells were rinsed three times with ice-cold PBS and lysed in a buffer containing 20 mM HEPES, pH 7.4, 2 mM EDTA, 0.3
M sucrose, 1.0% NP-40, 0.1% sodium dodecyl sulfate, 5 μg/ml antipain, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 4 mM benzamidine, 5 μg/ml chymostatin, 5 μg/ml pepstatin A, and 0.105 M 4(2-aminoethyl)-benzene sulfonyl fluoride. Total protein was determined using Coomassie Plus reagent (Pierce, Rockford, IL), based on Bradford’s colorimetric method. Results were expressed in picomoles of NO per milligram of protein after 2-min stimulation.

Statistics. Data are reported as means ± SE. They were evaluated by t-test or exact nonparametric Wilcox two-sample test as appropriate. To compare the relaxation-response curves, a four parametric logistic model was fit to the dose-response curves. All statistical analyses were performed by the Biostatistics Department at Henry Ford Hospital. P < 0.05 was considered significant.

RESULTS

To make sure the mouse thoracic aorta is a valid model to test our hypothesis, we first investigated whether AQP-1 is expressed in both endothelial and vascular smooth muscle cells of these vessels by Western blot. Using whole cell homogenates, we found that both endothelial cells and vascular smooth muscle cells isolated from thoracic aortas of control mice expressed AQP-1. However, its relative abundance was 20.3 times higher in vascular smooth muscle cells compared with endothelial cells (P < 0.0005; n = 4; Fig. 1). The presence of AQP-1 detected in endothelial cells was not due to contamination with vascular smooth muscle cells since vascular smooth muscle cell α-actin was only detected in isolated vascular smooth muscle cells and not endothelial cells. In addition, NOS3 was only detected in isolated endothelial cells but not vascular smooth muscle cells (Fig. 1). These data indicate that AQP-1 is present in both cell types in the mouse thoracic aorta, and thus this vessel is a valid model to test our hypothesis.

To investigate whether transport of NO by AQP-1 plays a role in endothelium-dependent relaxation, we first studied the ability of NO released by endothelial cells to relax intact aortic rings. NO release by endothelial cells was stimulated by adding increasing concentrations of acetylcholine to the bath. We found that the relaxant effect of acetylcholine was diminished in aortic rings isolated from AQP-1−/− mice compared with controls at all concentration tested (10−9–3 × 10−6 M) being the maximum relaxation reduced by 30% in aortas from AQP-1−/− mice compared with control mice (P < 0.05; n = 5–7; Fig. 2). However, the EC50 values were similar between both curves (2.48 ± 0.13 × 10−8 for CD1 and 4.05 ± 0.12 × 10−8 M for AQP-1−/−; not significant). These data suggest that in the mouse thoracic aorta, the ability of acetylcholine to induce endothelium-dependent relaxation is impaired by the absence of AQP-1.

The ability of acetylcholine to cause relaxation of the thoracic aorta is mainly attributed to stimulation of NO release by endothelial cells. However, in certain blood vessels other mechanisms have been shown to be the mediators of acetylcholine-induced relaxation such as cytochrome P-450 epoxygenase products (15, 66) and endothelium-dependent hyperpolarizing factor. To make sure that in our experiments all the effects of acetylcholine were mediated by NO, we tested the effect of the NOS inhibitor l-NAME on acetylcholine-induced relaxation. l-NAME (1 mM) completely abolished the relaxation induced by acetylcholine (10−9–10−6 M) in aortic rings from both control and AQP-1−/− mice (maximum relaxation induced by 1 μM acetylcholine was −4.3 ± 2.5 and 5.4 ± 2.7% for AQP-1−/− and CD1 mice, respectively). Thus the mechanism whereby acetylcholine relaxes aortic rings is exclusively NO dependent.

The genetic background of AQP-1−/− mice is primarily CD1 with a small percentage of C57Bl/6J mixed in. To make sure our results were not due to strain differences in the response to acetylcholine, we measured endothelium-dependent relaxation in aortic rings from CD1 and C57Bl/6J mice. We found that maximum relaxation occurred at 3 μM acetylcholine for both CD1 (17.2 ± 1.1%) and C57Bl/6J (11.5 ± 6.2%; n = 3). Additionally, we found no differences in the EC50 for the concentration-response curve to acetylcholine between strains. Because there were no differences and, the AQP-1−/− are mostly on a CD1 background, we used CD1 as controls.

NO released by endothelial cells must enter the vascular smooth muscle cells to activate soluble guanylate cyclase and cause relaxation. The impaired endothelium-dependent relaxation found in aortic rings from AQP-1−/− mice may be due to 1) alterations in the signaling cascade beyond NO, 2) diminished NO influx into vascular smooth muscle cells, and/or 3) reduced NO efflux out of endothelial cells.

To investigate whether the differences we found in the ability of endogenous NO to relax aortic rings isolated from AQP-1−/− mice were due to an impairment of the signaling cascade beyond NO, we next investigated the ability of intra-
The EC50 values for Ach were 2.48 μM. Delivery of NO did not differ between AQP-1 aortic rings, the relaxation responses induced by intracellular NO were the same in both strains (Fig. 4). The EC50 values for Ach were 2.48 ± 0.02 μM. In controls (n = 6), the relaxation response to NO was the same in both strains (Fig. 4). The maximum relaxation to the highest dose of Ach was reduced by 30% in AQP-1 control (CD1) and aquaporin-1 knockout mice (AQP-1/−/−). The maximum relaxation to the highest dose of Ach was reduced by 30% in AQP-1 knockout mice compared with controls (6.30 ± 0.08 × 10−7 M; P < 0.0001). To investigate whether the reduced ability of spermine NONOate to relax aortic rings from AQP-1/−/− mice is due to reduced NO transport, we directly measured the rate of NO influx into isolated vascular smooth muscle cells from mouse thoracic aortas using fluorescence microscopy. NO influx was initiated by generating a 5-μM NO concentration gradient using the NO donor spermine NONOate. In isolated vascular smooth muscle cells, the rate of NO influx was 0.17 ± 0.02 fluorescence units/s for cells from controls and 0.07 ± 0.01 fluorescence units/s for cells from AQP-1/−/−, 60% lower (P < 0.0002; n = 6–8; Fig. 5). These data suggest that reduced AQP-1-dependent NO transport into vascular smooth muscle cells accounts, at least in part, for the diminished ability of extracellular NO to cause relaxation in AQP-1/−/−.

To investigate whether reduced NO transport out of endothelial cells could account, at least in part, for the impaired endothelium-dependent relaxation observed in AQP-1/−/−, we measured NO release by primary cultures of endothelial cells isolated from the thoracic aorta. In response to 1 μM acetylcholine, NO released by confluent endothelial cells from control mice was 96.2 ± 17.7 pmol NO/mg protein after 2 min.

cellular NO and cGMP to cause vasorelaxation. NO was delivered intracellularly using the NO donor sodium nitroprusside (10−10–3 × 10−7 M). This donor needs to be metabolized by the cells to release NO (4, 20, 44). In endothelium-denuded aortic rings, the relaxation responses induced by intracellular delivery of NO did not differ between AQP-1/−/− and controls (n = 6; Fig. 3). Next, we studied the ability of intracellular cGMP to cause vasorelaxation. Intracellular levels of cGMP were increased by treating rings with the phosphodiesterase 5 (PDE5) inhibitor 4-[(3, 4-(methylenedioxy)benzyl) amino]-6-methoxyquinazoline (10−7–3 × 10−5 M). In endothelium-denuded aortic rings, the relaxation responses induced by intracellular cGMP did not differ between AQP-1/−/− and controls (n = 4; Fig. 3). These data suggest that the ability of a given amount of intracellular NO and intracellular cGMP to activate the signaling cascade leading to relaxation is intact in the thoracic aorta of AQP-1/−/− mice. Thus the impaired endothelium-dependent relaxation in AQP-1/−/− mice is not due to alterations in the signaling cascade beyond NO.

Because vascular smooth muscle cells express 20 times more AQP-1 than endothelial cells, we next investigated whether reduced NO influx into vascular smooth muscle cells could account for the impaired endothelium-dependent relaxation in aortas isolated from AQP-1/−/− mice. For this, we studied the ability of exogenous NO to relax endothelium-denuded aortic rings. NO was added to the bath using increasing concentrations of the NO donor spermine NONOate (3 × 10−8–3 × 10−5 M), which spontaneously releases NO primarily into the extracellular environment (31, 58). We found that in endothelium-denuded rings, the cumulative relaxation-response curve to extracellular NO was shifted to the right in thoracic aortas isolated from AQP-1/−/− mice compared with controls (P < 0.0001, n = 5–6). The maximum relaxation response to NO was the same in both strains (Fig. 4). The EC50 for NO was higher for aortic rings isolated from AQP-1/−/− compared with controls (6.30 ± 0.08 × 10−7 M; P < 0.05). These data suggest that in the endothelium-denuded mouse thoracic aorta, the ability of extracellular NO to cause relaxation is diminished by the absence of AQP-1.
In contrast, NO released by endothelial cells from AQP-1−/− was 41.9 ± 13.4 pmol NO/mg protein, 56% less (P < 0.04; n = 5; Fig. 6). These data suggest that reduced AQP-1-dependent NO transport out of endothelial cells could partially account for the impaired endothelium-dependent relaxation in the AQP-1−/−.

Impaired NO efflux in endothelial cells from AQP-1−/− could be due to reduced NOS3 expression. Thus we measured NOS3 expression by endothelial cells isolated from both control and AQP-1−/− mice by Western blot. We found that NOS3 was 1.33 ± 0.29 O.D. units for controls and 3.84 ± 0.76 O.D. units for AQP-1−/−, a 188% increase (P < 0.01; n = 6; Fig. 7). These data suggest that the ability of endothelial cells to produce NO is enhanced in thoracic aortas of AQP-1−/−. Thus reduced NO production is not likely to account for impaired endothelium-dependent relaxation in AQP-1−/− mice.

Since NOS3 is increased in endothelial cells of AQP-1−/− mice, we next investigated whether superoxide production as a result of NOS3 uncoupling could account for the reduced ability of endogenous NO to cause relaxation. To do this, we tested whether superoxide scavenging could reverse the re-

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**Fig. 4.** Relaxant effect of extracellular NO on endothelium-denuded aortic rings from control (CD1) and AQP-1−/− mice. SPM, spermine NONOate (an NO donor that spontaneously releases NO into the bath). P < 0.0001 indicates that the curves are different. The EC50 values for SPM were 1.47 ± 0.02 × 10−7 for CD1 vs. 6.30 ± 0.08 × 10−7 for AQP-1−/− (P < 0.05; n = 5–6).

**Fig. 5.** NO influx by VSMC isolated from thoracic aortas of control (CD1) and AQP-1−/− mice. Top: representative traces of NO influx. The initial rate of increase in fluorescence (typically 50–100 s) was used to calculate the rate of NO influx. The line plotted through the data represents the initial rates. Bottom: mean data for initial rates of NO influx (P < 0.0002 vs. CD1; n = 6–8).

**Fig. 6.** Effect of 1 μM acetylcholine on NO release in confluent endothelial cells isolated from thoracic aortas of control (CD1) and AQP-1−/− mice (P < 0.04 vs. CD1; n = 5).

**Fig. 7.** Mean data and representative Western blot showing NOS3 expression by endothelial cells isolated from thoracic aortas of control (CD1) and AQP-1−/− mice. P < 0.01 vs. CD1; n = 6.
duced ability of acetylcholine to cause relaxation in the AQP-1−/− mice. We found that, in the presence of the superoxide scavenger Tempol (100 μM), the relaxant effect of acetylcholine was diminished in aortic rings isolated from AQP-1−/− mice compared with controls at all concentration tested (10−9–3 × 10−6 M) being the maximum relaxation reduced by 36% in aortas from AQP-1−/− compared with controls (P < 0.02; n = 4; Fig. 8). Thus NOS3 uncoupling does not account for the impaired endothelium-dependent relaxation in AQP-1−/−.

**DISCUSSION**

We believe our data demonstrate for the first time a physiological role for AQP-1-dependent transport of NO. We found that NO transport into vascular smooth muscle cells and out of endothelial cells is diminished in cells lacking AQP-1. As a result, NO-dependent relaxation is impaired in thoracic aortas from AQP-1−/− mice. We conclude that transport of NO via AQP-1 is essential for full expression of endothelium-dependent relaxation.

Since we demonstrated that in transfected cultured cells and reconstituted lipid vesicles AQP-1 transports NO across the lipid bilayer (24), we questioned whether transport of NO by AQP-1 is essential for full expression of endothelium-dependent relaxation.

We first investigated whether alterations in the signaling cascade beyond NO is altered, since we demonstrated that in transfected cultured cells and reconstituted lipid vesicles AQP-1 transports NO across the lipid bilayer (24); therefore, by using 5 μM NO to initiate influx because we previously showed that transport of NO by AQP-1 saturates at 3 μM NO (24); then, we were sure we were measuring the maximum rate of NO transport by AQP-1. We found that the rate of NO influx was decreased by 62% in vascular smooth muscle cells isolated from AQP-1−/−, indicating that AQP-1 transports NO into vascular smooth muscle cells. Our data suggest that reduced transport of NO into vascular smooth muscle cells could account, at least in part, for the reduced ability of acetylcholine-stimulated NO production by endothelial cells to relax aortic rings isolated from AQP-1−/− mice. However, reduced release of NO out of endothelial cells could also account for our data.

To investigate whether AQP-1 transports NO into vascular smooth muscle cells, we directly measured NO influx into vascular smooth muscle cells of wild-type and AQP-1−/− mice. We used 5 μM NO to initiate influx because we previously showed that transport of NO by AQP-1 saturates at 3 μM NO (24); therefore, by using 5 μM, we were sure we were measuring the maximum rate of NO transport by AQP-1. We found that the rate of NO influx was diminished by 62% in vascular smooth muscle cells isolated from AQP-1−/−, indicating that AQP-1 transports NO into vascular smooth muscle cells. Our data suggest that reduced transport of NO into vascular smooth muscle cells could account, at least in part, for the reduced ability of acetylcholine-stimulated NO production by endothelial cells to relax aortic rings isolated from AQP-1−/− mice. However, reduced release of NO out of endothelial cells could also account for our data.

To investigate whether AQP-1-dependent transport out of endothelial cells could account for the reduced ability of acetylcholine to relax intact aortic rings from AQP-1−/− mice, we measured NO released by endothelial cells upon stimulation with 1 μM acetylcholine. We chose this concentration because it produced the maximum relaxant effect in aortic rings from both wild-type and AQP-1−/−. Our data indicate that in response to equal amounts of acetylcholine, NO released by endothelial cells isolated from AQP-1−/− is reduced by 56%. Thus this could be at least partly responsible for the impaired endothelium-dependent relaxation in AQP-1−/−. However, it is important to note that while it appears that release of NO from endothelial cells is also impaired in AQP-1−/− and that this could account for the 30% blunting of maximum endothelium-dependent relaxation, several other possible explanations exist. These include a decrease in acetylcholine receptors, a decrease in G protein

![Fig. 8. Effect of the superoxide scavenger Tempol (100 μM) on Ach-induced relaxation of intact aortic rings from control (CD1) and AQP-1−/− mice. P < 0.05 indicates that the curves are different. The maximum relaxation to the highest dose of Ach was reduced by 36% in AQP-1−/− (P < 0.02 vs. CD1; n = 4).](http://ajprenal.physiology.org/)

**Fig. 8.** Effect of the superoxide scavenger Tempol (100 μM) on Ach-induced relaxation of intact aortic rings from control (CD1) and AQP-1−/− mice. P < 0.05 indicates that the curves are different. The maximum relaxation to the highest dose of Ach was reduced by 36% in AQP-1−/− (P < 0.02 vs. CD1; n = 4).
expression, and/or G protein activation. Exhaustive testing of all such possibilities is clearly beyond the scope of this manuscript. It appears unlikely that the effects are due to changes in NOS3, because we found that NOS3 expression was increased by 188% in endothelial cells isolated from AQP-1−/− compared with wild-type. The fact that endothelial cells from AQP-1−/− exhibited higher levels of NOS3 may be due to an adaptive mechanism developed by these animals to compensate for the diminished effects of NO within the vasculature caused by systemic lack of AQP-1.

The concept that NO freely diffuses across lipid bilayers was originally based on the partition coefficient of NO between hydrophobic phases and water. The partition coefficient of NO in 1-octanol and liposomes relative to water was found to be 6.5 (42) and 4.4 (46). It has been assumed that because solubility of NO is higher in hydrophobic phases, NO freely diffuses across cell membranes. However, partition coefficients are measured at equilibrium and do not provide any information concerning the rate at which equilibrium is achieved. Additionally, due to the higher solubility of NO in lipids, it is energetically unfavorable for NO to leave the membrane. Thus one cannot predict transmembrane rates of NO flux based on partition coefficients.

In 1996, Subczynski et al. (59) calculated the permeability coefficient for NO across membranes based on profiles of local NO diffusion concentration products in synthetic phospholipid bilayers using lipid-soluble NO spin labels and electron paramagnetic resonance. They reported that the lipid bilayer represents no barrier to the diffusion of NO. However, several approximations and assumptions entered into their calculations. Thus the apparent permeability values they obtained are not likely to reflect the real permeability of NO across cell membranes. For instance, the diffusion coefficient of NO in the lipid bilayer was not measured. Instead, the authors assumed that the diffusion coefficient for NO in the lipid phase was the same as in water. However, the diffusion coefficient of NO is reportedly five times lower in lipids with respect to water when measured by fluorescence quenching of derivatives incorporated at different positions in the membrane (12, 62). Such a difference on its own could cause a significant overestimation of the permeability coefficient. Similarly, it was assumed that the polar heads of phospholipids offer no resistance to the permeability of NO (13), and thus their calculation would reflect permeability of NO “within” but not “across” the membrane. Finally, experimental measurements of the NO diffusion concentration product were performed under non-physiological conditions. The concentration of NO was 2 mM, at least 1,000 times that produced by endothelial cells; the experiments were carried out at 25°C, a temperature below the transition phase of cell membranes; and the measurements were done in synthetic lipid bilayers. Under physiological situations, the protein content of cell membranes would decrease the partition coefficient of NO into the lipid bilayer (57). In contrast to calculated NO permeabilities, we recently demonstrated directly that the cell membrane is a significant barrier to NO diffusion and that AQP-1 facilitates NO transport (24).

Our data indicate that AQP-1 facilitates diffusion of NO across the mammalian cell membrane. However, free diffusion of NO driven by NO concentration gradients across cell membranes would still be expected to occur, although at lower rates (24). This raises the question of why, even at the higher levels of NO induced by the highest doses of acetylcholine, free diffusion of NO is not sufficient to completely relax aortic rings from AQP-1−/− mice. Because NO undergoes rapid degradation (42), intracellular NOS3-derived NO concentrations may not build up enough to reach the vascular smooth muscle cells by simply equilibrating across cell membranes. As a result, the amount of NO that crosses the cell membrane by free diffusion is not sufficient to cause full relaxation. In contrast, in the presence of AQP-1, NO produced by the cell is rapidly extruded via this channel; consequently, less degradation would occur, and more NO could cross the cell membrane. Given that there was a 30% reduction in response to acetylcholine in AQP-1−/−, one might well ask why this difference was not seen in the experiments involving spermine NONOate. The most likely explanation is that spermine NONOate produces a higher concentration of NO and that the concentration of NO is constant for a longer period of time (31, 58) than when the NO is produced by endothelial cells. Thus the fraction of the total amount of NO produced that is degraded is smaller and diffusion of NO can occur rapidly enough to produce maximum relaxation.

NO is an important regulator of blood pressure. In peripheral blood vessels, NO dilates blood vessels and lowers total peripheral resistance. Additionally, NO helps maintain the low renal vascular resistance characteristic of the kidney by dilating pregglomerular arterioles. It also regulates ion and solute transport along the nephron, mediates pressure-natriuresis, and modulates tubuloglomerular feedback (7, 25, 30, 35, 39–41, 43, 54, 61). This raises the question of whether deletion of AQP-1 alters blood pressure. Interestingly, AQP-1−/− mice are not hypertensive; if anything, mean blood pressure is slightly reduced (unpublished data from this laboratory). Several possibilities can explain this. First, these mice are polyuric due to disruption of the countercurrent multiplication mechanism, which in turn is attributable to the lack of AQP-1 in the thin descending limb and descending vasa recta within the kidney (10, 37, 52). The antihypertensive effects of polyuria would be expected to overwhelm the increased vascular tone resulting from the reduced effects of NO in the vasculature. Second, the rapid scavenging of NO by its reaction with hemoglobin would be expected to be reduced in the knockouts because of the lack of AQP-1 in the membrane of red blood cells. This would result in enhanced NO concentrations, which in turn would increase diffusion, thus enhancing the effects of NO in vascular smooth muscle cells. Third, other modulators of vascular tone, such as prostaglandins (5, 11, 21, 63), carbon monoxide (CO) (8, 29, 67, 68), cytochrome P-450 epoxyene products (15, 27, 66), and endothelium-dependent hyperpolarizing factor (48, 64), could be upregulated in the knockouts, mitigating the reduced vasodilator effect of NO in the vasculature. Finally, other aquaporins may compensate for the lack of AQP-1 within the vasculature.

We conclude that 1) AQP-1 transports NO into vascular smooth muscle cells, 2) AQP-1 transports NO out of endothelial cells, 3) the membrane of mammalian cells is a significant barrier to diffusion of NO, 4) transport of NO by AQP-1 into vascular smooth muscle cells and out of endothelial cells is essential for full expression of endothelium-dependent relaxation, and 5) transport of NO by AQP-1 enhances its physiological effects.
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