NOS3 Deletion in Cav1 Deficient Mice Decreases Drug Sensitivity to a Nitric Oxide Donor and Two Nitric Oxide Synthase Inhibitors

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PURPOSE. This study aims to investigate the pharmacologic consequence of genetic deletion of nitric oxide synthase 3 (NOS3) in caveolin 1 (Cav1)−/− mice (double knockout [DKO]) in response to a nitric oxide (NO) donor and two NO inhibitors.

METHODS. NO donor sodium nitroprusside (SNP; 10–40 mg/mL), NO inhibitor L-NG-nitroarginine methyl ester (L-NAME; 10–200 μM), and cavatatin (10–75 μM) was administered topically to the eye while the contralateral eyes were vehicle controls. Intraocular pressure (IOP) was measured in both eyes by tonometry. Cyclic guanosine monophosphate (cGMP) level in outflow tissue was measured by ELISA assay. Protein expression were analyzed by western blot.

RESULTS. Inducible NOS (iNOS) expression significantly increased in the DKO mice compared with the wild type (WT), Cav1 knockout (Cav1 KO), and NOS3 KO mice. In contrast to WT, Cav1 KO and NOS3 KO mice, SNP concentration of up to 30 mg/mL did not significantly affect IOP in DKO mice. However, higher concentration (40 mg/mL) SNP significantly reduced IOP by 14% (n = 8, P < 0.01). Similarly, only 200 μM L-NAME produced a significant increase in IOP (n = 10, P < 0.05). Cavatatin did not significantly change IOP in DKO and NOS3 KO mice. cGMP activity in DKO mice was significantly lower than Cav1 KO mice (n = 4, P < 0.05).

CONCLUSIONS. In conclusion, our results demonstrated that genetic deletion of NOS3 in Cav1 deficient mice resulted in reduced sensitivity to the NO donor SNP and the two NO inhibitors possibly due to compromised NOS and cGMP activity.

Keywords: NOS3, Cav1, nitric oxide, intraocular pressure, glaucoma

Endothelial nitric oxide synthase (eNOS) and caveolin 1 (Cav1) are important molecules in regulating intraocular pressure (IOP).1–8 Nitric oxide synthase 3 (NOS3) and Cav1 gene polymorphism were associated with the risk of developing primary open angle glaucoma (POAG) and increased intraocular (IOP).9–16 Previous study showed that the deletion of either NOS3 or Cav1 gene results in IOP elevation.2,4,17 However, interestingly, the deletion of NOS3 in Cav1 deficient mice restored outflow function and IOP.18

Cav1 interacts with a variety of signaling molecules, for example, epidermal growth factor receptor (EGFR), c-Src, phosphoinositide 3-kinase (PI3K), and Gi, among which eNOS is one of the most important molecules.19,20 The binding of Cav1 to eNOS via its scaffold domain is an important negative regulator of eNOS activity.21 When Cav1 was absent, eNOS was activated and IOP elevated.22 In endothelial cells, it was demonstrated that Cav1 might regulate eNOS and Src signaling through feedback and feed-forward mechanisms.22–24 The reduced Cav1 expression was linked to eNOS-dependent reactive oxygen species production and disruption of endothelial monolayer integrity, which could cause pulmonary arterial hypertension.25 In response to stimuli, NO is produced via eNOS activation.3,22–24 The binding of Cav1 to eNOS prevents unfettered activation of eNOS and resultant oxidative stress via peroxynitrite production that can cause cell damage in the vasculature and the aqueous humor outflow tissue of the eye.5,26–28 This mechanism appears critical in maintaining endothelial cell homeostasis. Interestingly, genetic deletion of NOS3 and Cav1 (double knockout [DKO]) mice prevented pulmonary hypertension, pulmonary vascular remodeling,26 completely reversed cardiac hypertrophy,29 lowered plaque formation in carotid artery transplantation,30 and more importantly here, it restored aqueous humor outflow function by wild type (WT) level.19

Sodium nitroprusside (SNP) can reduce IOP in NOS3−/− and Cav1−/− mice, which suggests that the deletion of either of the genes does not affect the NO-cGMP downstream signaling. In contrast, NOS3 and Cav1 DKO mice are unresponsive to the conventional dose of NO donor. If this is the case, glaucoma patients with the genetic abnormality in both genes may be...
insensitive to certain drug treatment. Therefore, it is important to know the pharmacological consequence of the genetic deficiency. This study aims to investigate the pharmacologic consequence of genetic deletion of NOS3 in Cav1KO mice in response to the NO donor SNP and the two NOS inhibitors systematically and to establish a dose-response relationship.

METHODS

Animals

Animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. NOS3 and Cav1 DKO mice were obtained by crossing NOS3+/− (stock number: 002684, Jackson Laboratory, Bar Harbor, ME, USA) and Cav1KO mice (stock number: 007083, Jackson Laboratory) of the same background (C57BL/6j) and purchased from Jackson Laboratory through the Model Animal Research Center at Nanjing University. Male mice aged between 8 and 10 weeks were used in this study. Control mice were the WT C57BL/6J littermates. Mice were genotyped according to the established protocol provided by Jackson Laboratory.19

Western Blot

Outflow tissue was dissected following an established method,3 which contained trabecular meshwork (TM), Schlemm’s canal (SC), and possibly some iris root. The tissue sample was lysed with radio immunoprecipitation assay solution. Fifty micrograms of proteins were loaded, which was separated by SDS-PAGE (10% or 12.5% acrylamide). Then the resolved proteins were transferred by electrophoresis to nitrocellulose membranes, which were blocked by 5% nonfat dry milk for 2 hours. The nitrocellulose membranes were probed with primary antibodies and peroxidase-liked secondary antibodies. Primary antibodies used included inducible NOS (iNOS) (1:1000, Abcam, Shanghai, China), neuronal NOS (nNOS) (1:1000, Abcam), and Caveolin 2 (Cav2) (1:1000, Abcam). The loading control was glyceraldehyde-3-phosphate dehydrogenase. Densitometry analysis was performed on the digital signals of the linear range of the x-ray film.

IOP Measurements in Mice

IOP was measured in both mouse eyes without anesthesia at the same time of day (10 to 11 AM) using rebound tonometry (TonoLab; ICare, Espoo, Finland). The mice were handled and acclimatized for IOP measurements for approximately 2 weeks before the experiments. The mouse stood relaxed while the researcher gently restrained the mouse body with one hand to keep it still, and IOP was measured with the other hand. IOP was measured three times, and the average was counted as a single measurement.

Topical Drug Application

One to 2 days before the pharmacology experiments, IOP of the four strains of mice were measured. SNP, L-NG-nitroarginine methyl ester (L-NAME), and cavintratin were dissolved in PBS and made into drug solutions. Mouse eyes were given topical applications of SNP (4 × 2 μL drops), L-NAME (4 × 2 μL drops), or cavintratin (4 × 2 μL drops) at 0, 0.5, 1, and 1.5 hours following established method.3 The contralateral eyes were treated with drug vehicle. Two sets of experiments were carried out testing a range of concentrations of the NO donor (SNP, 10, 20, 30, and 40 mg/mL) and NOS inhibitors (L-NAME, 10, 100, and 200 μM or cavintratin 10, 50, and 75 μM). IOP was measured in both eyes before drug treatment and 1.5 hours after the last drug treatment.

Enzyme-Linked Immunosorbent Assay

The levels of cGMP in the outflow tissue were determined using commercially available kits (Cyclic GMP XP Assay Kit, Cell Signaling Technology, Shanghai, China) according to the manufacturer’s instructions. OD450 nm readings were averaged from four duplicate wells.

Statistics

Statistical analysis was performed using SPSS 21.0 (IBM, Chicago, IL, USA). Data normality was first tested. IOP of four strains of mice was analyzed by nonparametric test. IOP of drug-treated eyes was analyzed by 2-way analysis of variance (ANOVA) followed by least significant difference (LSD) test for multiple comparisons. Western blot densitometry data were analyzed by nonparametric Mann-Whitney independent sample t-test. cGMP assay results were analyzed by nonparametric Kruskal-Wallis ANOVA test. In all cases, differences were considered significant at P < 0.05.

RESULTS

NOS and Cav Expressions

The lack of NOS3 and Cav1 gene in the NOS3 KO and Cav1 KO mice were demonstrated previously.19 By crossing the two strains of mice, DKO mice have no expression of either NOS3 or Cav1 (Supplementary Fig. S1). We first determined NOS isoforms expression and Cav2 expression in DKO mice. NOS3 expression increased in DKO mice compared with WT, Cav1 KO, and NOS3 KO mice (Fig. 1). However, the nNOS
WT mice. Interestingly, IOP measurements in the DKO mice (10 ± 0.9 mm Hg, n = 12) were not significantly different from that of WT mice. In Cav1 KO mice, Cav2 protein was absent (Fig. 1). In comparison, WT mice expressed Cav2 in the TM and SC tissue (Fig. 1).

**Cav1 and eNOS Independently Regulate IOP**

Four mouse strains were used to investigate the role of Cav1 and eNOS in regulating IOP. Figure 2 demonstrates that IOP was significantly increased in NOS3 KO mice (15 ± 1.9 mm Hg, n = 12) and Cav1 KO mice (16 ± 1.5 mm Hg, n = 12) when compared with WT mice (11 ± 1.5 mm Hg, n = 12). Interestingly, IOP measurements in the DKO mice (10 ± 0.9 mm Hg, n = 12) were not significantly different from that of WT mice.

**Response to a NO Donor SNP**

Next, we investigated the dose-response relationship to the NO donor SNP in WT, Cav1 KO, NOS3 KO, and DKO mice (Fig. 3A). A summary of IOP and the percentage change of IOP is in Supplementary Table S2. In NOS3 KO mice, SNP were 6.2, 3.7, and 4.0 mm Hg (n = 6) for each group; Fig. 3A); the percentage of IOP reduction was 11% to 18% (Supplementary Table S2). In Cav1 KO mice, ΔIOP of 20, 30, and 40 mg/mL SNP were 2.5, 1.9, and 3.0 mm Hg (n = 6 for each group; Fig. 3A); the percentage of IOP reduction was 14% to 24% (Supplementary Table S2). In DKO mice, ΔIOP of 20, 30, and 40 mg/mL SNP were 6.2, 3.7, and 4.0 mm Hg (n = 6 for each group; Fig. 3A); the percentage of IOP reduction was 25% to 38% (Supplementary Table S2). In DKO mice, 10 to 30 mg/mL SNP had no significant effect on IOP; 40 mg/mL SNP significantly lowered IOP from 12 to 10 mm Hg (ΔIOP = 1.7 mm Hg, P < 0.01, n = 8); the percentage IOP reduction was 14% (Supplementary Table S2). Ten milligrams per milliliter SNP did not significantly change IOP in any of the four mouse strains of WT (n = 10), Cav1 KO (n = 6), NOS3 KO (n = 6), and DKO mice (n = 6, P > 0.05; Fig. 3A).

**Response to the NOS Inhibitor L-NAME**

Similarly, to determine the dose-response to NOS inhibitor, we topically applied different concentrations of L-NAME solution (10, 100, and 200 μM) to the mouse eyes (Fig. 3B). Ten and one hundred micromolars L-NAME did not affect IOP (10 μM, n = 8; 100 μM, n = 15; P > 0.05); 200 μM L-NAME significantly increased IOP in DKO mice (n = 10, P < 0.05). In contrast, both 100 and 200 μM L-NAME significantly increased IOP in WT, Cav1 KO, and NOS3 KO mice (P < 0.05, WT, 100 μM, n = 6, 200 μM, n = 6; Cav1, 100 μM, n = 6, 200 μM, n = 6; NOS3 KO, 100 μM, n = 4, 200 μM, n = 6). In WT mice, ΔIOP of 100 and 200 μM L-NAME were 2.5 and 1.5 mm Hg (Fig. 3B); the percentage of IOP elevation was 12% to 22% (Supplementary Table S2). In Cav1 KO mice, ΔIOP of 100 and 200 μM L-NAME were 2.2 and 5 mm Hg (Fig. 3B); percentage of IOP elevation was 14% to 19% (Supplementary Table S2). In NOS3 KO mice,
KO mice (Fig. 3C). Unexpectedly, 50 \(\text{L-NAME} \) did not cause any significant change in IOP in any of the four mouse strains of mice (WT, \( n = 6 \); Cav1 KO, \( n = 6 \); NOS3 KO, \( n = 5 \); DKO mice, \( n = 8 \); \( P > 0.05 \), Fig. 3B).

**Response to an eNOS Inhibitor Cavtratin**

Ten, fifty, and seventy-five micromolars of cavtratin significantly increased IOP in WT mice (\( P < 0.05 \), \( n = 6 \)), so did 75 \(\text{L-NAME} \) in Cav1 KO mice (Fig. 3C; Supplementary Table S2). In DKO and NOS3 KO mice, cavtratin did not significantly change IOP (DKO: 10 \(\mu M \), \( n = 6 \); 50 \(\mu M \), \( n = 6 \); 75 \(\mu M \), \( n = 6 \); NOS3 KO: 10 \(\mu M \), \( n = 6 \); 50 \(\mu M \), \( n = 6 \); 75 \(\mu M \), \( n = 5 \); \( P > 0.05 \), Fig. 3C). Unexpectedly, 50 \(\mu M \) cavtratin significantly increased IOP in WT mice (\( \Delta \text{IOP} = 2.4 \text{ mm Hg} \); \( n = 10 \), \( P < 0.05 \); Fig. 3B); the percentage of IOP elevation was 21\% (Supplementary Table S2). Low concentration of L-NAME (10 \(\mu M \)) did not cause any significant change in IOP in any of the four mouse strains of mice (WT, \( n = 6 \); Cav1 KO, \( n = 6 \); NOS3 KO, \( n = 5 \); DKO mice, \( n = 8 \); \( P > 0.05 \), Fig. 3B).

**cGMP activity**

cGMP in outflow tissue was 0.04 ± 0.005, 0.08 ± 0.023, 0.02 ± 0.007, and 0.01 ± 0.002 nmol/g in WT, Cav1 KO, NOS3 KO, and DKO mice, respectively. cGMP in the outflow tissue of the DKO mouse was significantly lower compared with Cav1 KO mice (Fig. 4). cGMP in DKO mice was lower than NOS3 KO and WT mice, but none reached the statistical significance level of 0.05.

**DISCUSSION**

The main finding of the present study is that Cav1 deletion in NOS3/−/− mice results in reduced IOP response to the NO donor SNP, the NO inhibitor L-NAME, and NOS3 inhibitor cavtratin, which may be due to compromised NOS and cGMP activity. Further, DKO mice expressed more iNOS and less eNOS than controls, which is similar to POAG patients whose iNOS was upregulated and eNOS was downregulated compared with normal control patients.6,28

In DKO mice, Cav1 KO diminished Cav2 protein expressions in these mice (Fig. 1). Cav1 was known to stabilize Cav2 protein levels.31,32 The absence of Cav2 in DKO mice is consistent with previous findings that Cav1 KO resulted in Cav2 absence in pulmonary vasculature.35 DKO mice exhibit normalized IOP, which is similar to WT mice (Fig. 2), is is this could be due to a combination of upregulation of iNOS (Fig. 1) and suppressed eNOS overactivation.18 The NO produced by iNOS can relax TM and SC endothelial cells.34

Among the four strains of mice, SNP induced IOP reduction was the smallest in DKO mice (Fig. 3A; Supplementary Table S2). The maximum IOP reduction in DKO mice is 14\% lower than WT, Cav1 KO, and NOS3 KO mice at the same concentration. This may suggest that the deletion of a single gene (NOS3 or Cav1) does not affect its IOP response to SNP. However, NOS3 knockout in Cav1 KO mice affected NO downstream signaling pathway and rendered it less sensitive to NO. Our data showed that cGMP level (indicating reduced sGC activity) in DKO mice was compromised (Fig. 4), and the vasodilation action of NO is mainly mediated through stimulation of cGMP, which may explain the reduced sensitivity to the NO donor SNP. It is interesting to note that, in Cav1 KO and DKO mice, the maximum IOP responses were produced by the highest dose of SNP (40 mg/mL) except for WT and NOS3 KO mice where the maximum IOP reduction was produced by 20 mg/mL SNP. This may suggest that in these mice, the feedback mechanism is at work to maintain IOP homeostasis. This is consistent with a study in rabbits that 0.1% SNP produced a higher level of IOP reduction 1 hour after drug application than 2% of SNP (0.1% and 2% SNP dosing is equivalent to 50 and 1000 \(\mu G \) SNP).35 In monkeys, 50 and 500 \(\mu G \) produced a similar magnitude of IOP reduction of between 10\% and 15\%.36

DKO mice showed significant IOP response only to the highest dose of L-NAME. The magnitude of IOP elevation is comparable with WT, Cav1 KO, and NOS3 KO mice (Fig. 3B). L-NAME elicited an increase in IOP in other species, for example, in rabbit37 and monkey.36 In DKO mice, although eNOS was absent, iNOS and nNOS were still present (Fig. 1). Therefore, L-NAME could have increased IOP by inhibiting iNOS and nNOS activity. The absence of eNOS in DKO mice may contribute to the reduced sensitivity to L-NAME.

DKO mice are unable to respond to cavtratin (Fig. 3C). Cavtratin is an eNOS-selective inhibitor, and it has been reported to decrease the outflow facility in WT and eNOS-GFPTg mice ex vivo.36 In our studies, cavtratin had no effect on IOP of DKO and NOS3 KO mice. This could be due to the lack of NOS3 gene in these strains of mice. We found a paradoxical lowering of IOP in the Cav1 KO mouse with 50 \(\mu M \) cavtratin and an increase of IOP in response to 75 \(\mu M \) cavtratin. The reason for this is unclear. We speculate that 50 \(\mu M \) cavtratin may have inhibited eNOS overactivation related TM/SC outflow tissue damage39,40 and therefore reduced IOP, but higher concentration of cavtratin further/over suppressed eNOS activity and NO production and led to IOP elevation. cGMP (indicating reduced sGC activity) in DKO mice was compromised (Fig. 4). The vasodilation action of NO is mainly mediated through stimulation of cGMP, which may explain the reduced sensitivity to the NO donor SNP in DKO mice compared with Cav1 KO mice. In mouse lung tissue, cGMP of DKO mice was significantly lower than Cav1 KO mice, which is consistent with our data in the outflow tissue. The lower level of cGMP in DKO compared with WT and NOS3 KO mice did not reach statistical significance of 0.05, and future investigations may consider involving a larger sample size.

Besides the NO signaling pathway, other important tuning pathways are interrelated to regulate IOP.41,42 The vital role of carbon monoxide (CO) has been confirmed in organisms...
lacking heme oxygenase-1 (gene name HMOX1), which is the enzyme that produces CO in neurons. NO has a tight relationship with the other gases such as CO and hydrogen sulfide (H₂S). CO, NO, and H₂S interacted in the modulation of cGMP in the retina. 43 H₂S inhibited NO-induced increases in cGMP but did not affect its own or CO-induced expression. 45 Furthermore, TM cell volume and contractility could be regulated by the NO/CO system. 41

Human genetic variation(s) in drug target may alter the therapeutic efficacy of drug, which may make people respond differently to the same medication. 44 Also, genetic variation(s) may alter the drug safety and increase the risk of adverse reactions. 45 It is estimated that an average of 3% of the population have receptors that contain mutations, which can alter the effect of medicine. 46 Pharmacogenomics and glaucoma have been studied extensively in recent years. For example, genetic variation is linked to differences in response to IOP-lowering drugs such as β-adrenergic antagonists 47–49 and prostaglandin analogs 50–52 Latanoprostene bunod marketed in 2017 under the brand name VYZULTA is the first NO-donor drug for the treatment of ocular hypertension and open-angle glaucoma. Although clinical trials have confirmed that it is a safe and effective drug, genetic variation(s) may affect how individuals respond to the same drug. The association of NOS3 polymorphisms with POAG had been reported in the Egyptian population, 53 Brazilian population, 54 and Pakistani population. 55 Cav1 polymorphisms showed significant association with POAG in Caucasians reported so far. 13 The minor allele frequency (MAF) of the two SNPs, rs1052990 and rs4236601, of Cav1 in different populations is 0.34 to 0.41 and 0.28 to 0.32, respectively. 13,14 The MAF of the two SNP of NOS3 in different populations, rs2070744 and rs1799983, is 0.11 to 0.35 and 0.08 to 0.42, respectively. 56 Future investigation in patients is needed to evaluate the pharmacogenomics of Cav1 and NOS3 in response to the new generation of NO donating glaucoma drugs.

In conclusion, genetic deletion of NOS3 in Cav1 deficient mice results in reduced drug sensitivity to the NO donor SNP and two NO inhibitors (L-NAME and cavtratin), which may be due to the altered NOS and NO downstream signaling in the conventional outflow tissue compared with Cav1 KO mice.

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