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

Maomao Song,1 Liping Li,1 Yuan Lei,1–3 and Xinghuai Sun1–4
1Department of Ophthalmology and Vision Science, Eye and ENT Hospital, Shanghai Medical College, Fudan University, Shanghai, China
2Key Laboratory of Myopia, Ministry of Health, Fudan University, Shanghai, China
3Shanghai Key Laboratory of Visual Impairment and Restoration, Eye and ENT Hospital, Shanghai Medical College, Fudan University, Shanghai, China
4State Key Laboratory of Medical Neurobiology, Institutes of Brain Science, Fudan University, Shanghai, China

Correspondence: Xinghuai Sun, Department of Ophthalmology, Eye and ENT Hospital of Fudan University, Shanghai 200031, China; xhsun@shmu.edu.
Yuan Lei, Research Centre, Eye and ENT Hospital of Fudan University, Shanghai 200031, China; lilian0167@hotmail.com.
MS and LL contributed equally to the work presented here and should therefore be regarded as equivalent authors.
Submitted: May 23, 2019
Accepted: August 21, 2019
Citation: Song M, Li L, Lei Y, Sun X. NOS3 deletion in Cav1 deficient mice decreases drug sensitivity to a nitric oxide donor and two nitric oxide synthase inhibitors. Invest Ophthalmol Vis Sci. 2019;60:4002–4007. https://doi.org/10.1167/iovs.19-27582

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 NOS inhibitors.

METHODS. NO donor sodium nitroprusside (SNP; 10–40 mg/mL), NOS inhibitor L-NG-nitroarginine methyl ester (L-NAME; 10–200 μM), and cavatrin (10–75 μM) was administered topically to the eye while the contralateral eyes were vehicle controls. Intracocular 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). Cavatrin 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 NOS inhibitors possibly due to compromised NOS and cGMP activity.

Keywords: NOS3, Cav1, nitric oxide, intraocular pressure, glaucoma
Jackson Laboratory. Genotyped according to the established protocol provided by the Model Animal Research Center at Nanjing University. Male (C57BL/6J) and purchased from Jackson Laboratory (stock number: 002684, Jackson Laboratory, Bar Harbor, ME, USA) and Cav1−/− mice (stock number: 007083, Jackson Laboratory) of the same background were used. The mice were 3 months of age in each experiment.

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 Cav1−/− 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 litters. Mice were genotyped according to the established protocol provided by Jackson Laboratory.

Western Blot

Outflow tissue was dissected following an established method, 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-like 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, Shanghai, China), neuronal NOS (nNOS) (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-NAM), and cavtratin were dissolved in PBS and made into drug solutions. Mouse eyes were given topical applications of SNP (4 × 2 μL drops), L-NAM (4 × 2 μL drops), or cavtratin (4 × 2 μL drops) at 0, 0.5, 1, and 1.5 hours after the last drug treatment. 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-NAM, 10, 100, and 200 μM or cavtratin 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.

Results

NOS and Cav Expressions

The lack of NOS3 and Cav1 gene in the NOS3 KO and Cav1 KO mice were demonstrated previously. 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. NOS expression increased in DKO mice compared with WT, Cav1 KO, and NOS3 KO mice, and did not express Cav2 compared with WT and NOS3 KO mice. However, the nNOS expression level was similar in all four strains of mice.
WT mice. Interestingly, IOP measurements in the DKO mice (10 ± 0.9 mm Hg, n = 12) compared with WT mice (11 ± 1.7 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.

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 = 6) and Cav1 KO mice (16 ± 1.5 mm Hg, n = 12) when compared with WT mice (11 ± 1.5 mm Hg, n = 12). Similarly, to determine the dose-response to NOS inhibitor, we investigated the dose-response relationship to the NO donor SNP in WT, Cav1 KO, NOS3 KO, and DKO mice (Fig. 3A). The right eye for each mouse was used. Error bar shows the standard deviation of the mean. WT, n = 12; Cav1 KO, n = 12; NOS3 KO, n = 12; DKO, n = 12. *P < 0.05.

Response to a NO Donor SNP

A summary of IOP and the percentage change of IOP is in Supplementary Table S2. In NOS3 KO mice, the IOP change (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 23% to 38% (Supplementary Table S2). In DKO mice, the IOP of 100 and 200 μM L-NAME significantly increased IOP (P < 0.05; WT, 20 mg/mL, n = 5; 50 mg/mL, n = 10; 40 mg/mL, n = 6; Cav1, 20 mg/mL, n = 6; 30 mg/mL, n = 6; 40 mg/mL, n = 6; NOS3 KO, 20 mg/mL, n = 6; 30 mg/mL, n = 6; 40 mg/mL, n = 6). In DKO mice, only the highest concentration (40 mg/mL) significantly lowered IOP (ΔIOP = 1.7 mm Hg, P < 0.01, n = 8). (B) Dose–response relationship of L-NAME. 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). Low concentration of L-NAME (10 μM) had no effect on IOP in any of the four mouse strains (P > 0.05; WT, n = 6; Cav1 KO, n = 6; NOS3 KO, n = 5; DKO, n = 8). (C) Dose–response relationship of cavatin. Ten, fifty, and seventy-five micromolars of cavatin significantly increased IOP in WT mice, but did not significantly change IOP in NOS3 KO and DKO mice. Fifty micromolars of cavatin significantly increased IOP in WT mice but lowers IOP in Cav1 KO mice (P > 0.05, n = 6). *P < 0.05, **P < 0.01.

Response to the NOS Inhibitor L-NAME

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. Twenty, thirty, and forty milligrams per milliliter SNP significantly lowered IOP in WT, Cav1 KO, and NOS3 KO mice; however, in DKO mice, only the highest dose of SNP lowered IOP. In WT mice, the IOP change (ΔIOP) of 20, 30, and 40 mg/mL SNP were 3.0, 1.6, and 2.1 mm Hg, respectively (n = 5, 10, 6, respectively; Fig. 3A); the percentage of IOP reduction was 14% to 24% (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 11% to 18% (Supplementary Table S2). In NOS3 KO 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 10 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).

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 3 mm Hg (Fig. 3B); percentage of IOP elevation was 14% to 19% (Supplementary Table S2). In NOS3 KO mice,
KO mice (IOP in WT mice, but none reached the statistical significance level of 0.05.

cGMP in DKO mice was lower than NOS3 KO: 10\(\mu\)M, \(n = 5\), DKO mice, \(n = 8\); \(P > 0.05\), Fig. 3B).

Response to an eNOS Inhibitor Cavatratin

Ten, fifty, and seventy-five micromolars of cavatratin significantly increased IOP in WT mice (\(P < 0.05\), \(n = 6\)), so did 75 \(\mu\)M cavatratin in Cav1 KO mice (Fig. 3C; Supplementary Table S2). In DKO and NOS3 KO mice, cavatratin 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 cavatratin significantly increased IOP in WT mice (\(\Delta\text{IOP} = 3.0 \text{ mm Hg}\)) but lowered IOP in Cav1 KO mice (\(\Delta\text{IOP} = 3.2 \text{ mm Hg}\)).

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 mice 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.

FIGURE 4. Comparison of cGMP concentration by ELISA between WT, Cav1 KO, NOS3 knockout (NOS3 KO), and NOS3 and Cav1 DKO mice. cGMP levels in DKO mice were compromised compared with Cav1 KO mice (mean ± SD, \(n = 4\), \(P < 0.05\)). Data are analyzed by nonparametric Kruskal-Wallis ANOVA test.

\(\Delta\text{IOP of 100 and 200 \(\mu\)M L-NAME were 2.0 and 2.8 mm Hg (Fig. 3B); the percentage of IOP elevation was 12\% to 17\% (Supplementary Table S2). In DKO mice, only the highest concentration (200 \(\mu\)M) L-NAME increased IOP (from 12 to 14 mm Hg, \(\Delta\text{IOP} = 2.4 \text{ mm Hg}, n = 10, P < 0.05\); 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).

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 hemostasis. 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). 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 mice (Fig. 3B). L-NAME elicited an increase in IOP in other species, for example, in rabbits and monkeys. 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 cavatratin (Fig. 3C). Cavatratin is an eNOS-selective inhibitor, and it has been reported to decrease the outflow facility in WT and eNOS-GFPTg mice ex vivo.38 In our studies, cavatratin 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 cavatratin and an increase of IOP in response to 75 \(\mu\)M cavatratin. The reason for this is unclear. We speculate that 50 \(\mu\)M cavatratin may have inhibited eNOS overactivation related TM/SC outflow tissue damage and therefore reduced IOP; but higher concentration of cavatratin 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. 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. H₂S inhibited NO-induced increases in cGMP but did not affect its own or CO-induced expression. Furthermore, TM cell volume and contractility could be regulated by the NO/CO system.

Human genetic variation(s) in drug target may alter the therapeutic efficacy of drug, which may make people respond differently to the same medication. Also, genetic variation(s) may alter the drug safety and increase the risk of adverse reactions. It is estimated that an average of 3% of the population have receptors that contain mutations, which can affect the effect of medicine. 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 and prostaglandin analogs. Latanoprostene bunod marketed in 2017 under the brand name VYZULTA is the first NO-donating 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 alter the drug safety and increase the risk of adverse reactions. It is estimated that an average of 3% of the population have receptors that contain mutations, which can affect the effect of medicine. 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 and prostaglandin analogs. Latanoprostene bunod marketed in 2017 under the brand name VYZULTA is the first NO-donating 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 NO35 polymorphisms with POAG had been reported in the Egyptian population, Brazilian population, and Pakistani population. Cav1 polymorphisms showed significant association with POAG in Caucasians reported so far. 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. The MAF of the two SNP of NO35 in different populations, rs2070744 and rs1799983, is 0.11 to 0.35 and 0.08 to 0.42, respectively. Future investigation in patients is needed to evaluate the pharmacogenomics of Cav1 and NO35 in response to the new generation of NO donating glaucoma drugs.

In conclusion, genetic deletion of NO35 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.

Acknowledgments

The authors thank W. Daniel Stamer, PhD, for his insightful comments on the manuscript and Dorette Ellis, PhD, for discussion on experiment design. We also thank Wang Yiqiang, PhD, for his generosity in sharing animal tissue and Xing Chao and Jufang Shi for their excellent assistance with the animal work. Supported by the National Science Foundation China (81100662, 81371015), Shanghai Municipal Health Bureau Young Outstanding Scientist Program (XYQ2013085), 211 Project of Fudan University (EHI518851), Scientific Research Foundation for the Returned Overseas Chinese Scholars (State Education Ministry), and Bright-Focus Foundation (G2018112).

Disclosure: M. Song, None; L. Li, None; Y. Lei, None; X. Sun, None

References


