

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 cavtratin (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 ($n = 10$, $P < 0.05$). Cavtratin 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

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.^{3,4,17} 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.³ In endothelial cells, it was demonstrated that Cav1 might regulate eNOS and Src signaling through feed-back and feed-forward mechanisms.²²⁻²⁴ The reduced Cav1 expression was linked to eNOS-dependent reactive oxygen species production and disruption of endothe-

lial 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.^{3,26-28} This mechanism appears critical in maintaining endothelial cell homeostasis. Interestingly, genetic deletion of NOS3 and Cav1 (double knockout [DKO] NOS3^{-/-}Cav1^{-/-}) 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 to wild type (WT) level.¹⁸

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 Cav1^{-/-} 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 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 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-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). 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 acclimated 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 cavtratin 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 cavtratin (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 cavtratin 10, 50, and 75 μM). IOP was

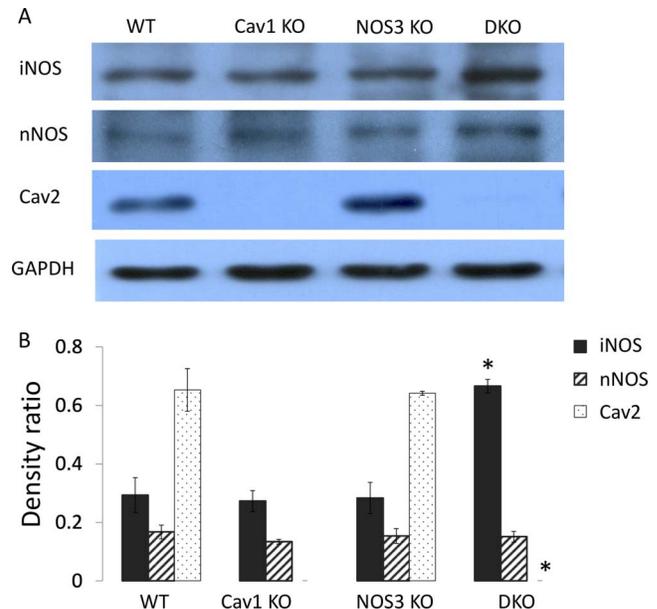


FIGURE 1. iNOS, nNOS, and Cav2 expression in WT, Cav1 KO, NOS3 knockout (NOS3 KO), and NOS3 and Cav1 DKO mice. (A) Representative blots showing DKO mice have increased expression of iNOS compared with WT, Cav1 KO, and NOS3 KO mice, and did not express Cav2 compared with WT and NOS3 KO mice. However, the nNOS expression level was similar in all four strains of mice. (B) Densitometric analyses of Western blots. Error bar shows the standard deviation of the mean. $n = 6$, $*P < 0.05$.

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. iNOS expression increased in DKO mice compared with WT, Cav1 KO, and NOS3 KO mice (Fig. 1). However, the nNOS

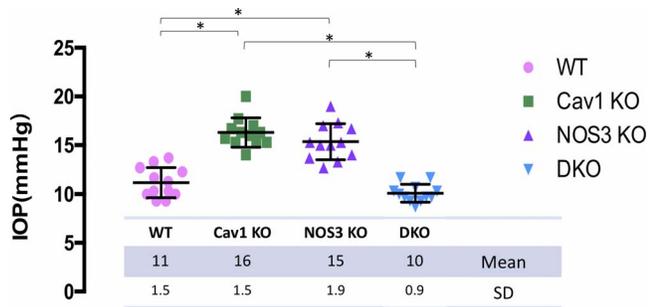


FIGURE 2. IOP of WT, Cav1 KO, NOS3 knockout (NOS3 KO), and NOS3 and Cav1 DKO mice. The right eye for each mouse was used. Error bar shows the standard deviation of the mean. WT, $n = 12$; Cav1 KO, $n = 12$; NOS3 KO, $n = 12$; DKO, $n = 12$, * $P < 0.05$.

expression level was similar in all four strains of mice (Fig. 1). 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 mice 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. 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 (Δ IOP) 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, Δ IOP 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, Δ IOP 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, 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 (Δ IOP = 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 μ M) to the mouse eyes (Fig. 3B). Ten and one hundred micromolars L-NAME did not affect IOP (10 μ M, $n = 8$; 100 μ M, $n = 15$; $P > 0.05$); 200 μ M L-NAME significantly increased IOP in DKO mice ($n = 10$, $P < 0.05$). In contrast,

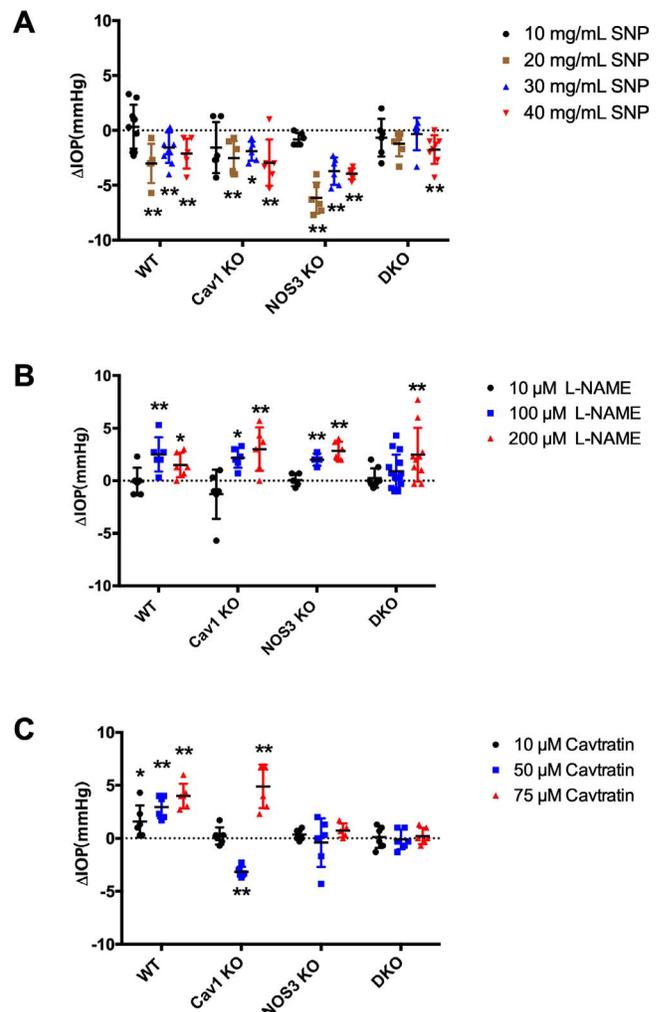


FIGURE 3. Dose-response relationship of SNP, L-NAME, and cavtratin in WT, Cav1 KO, NOS3 knockout (NOS3 KO), and NOS3 and Cav1 DKO mice. (A) Dose-response relationship of SNP. Twenty, thirty, and forty milligrams per milliliters of SNP significantly reduced IOP in WT, Cav1 KO, and NOS3 KO mice ($P < 0.05$; WT, 20 mg/mL, $n = 5$; 30 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 (Δ IOP = 1.7 mm Hg, $P < 0.01$, $n = 8$). (B) Dose-response relationship of L-NAME. In WT, Cav1 KO, and NOS3 KO mice, 100 and 200 μ M L-NAME significantly increased IOP ($P < 0.05$, WT, 100 μ M, $n = 6$, 200 μ M, $n = 6$; Cav1, 100 μ M, $n = 6$, 200 μ M, $n = 6$; NOS3 KO, 100 μ M, $n = 4$, 200 μ M, $n = 6$). Low concentration of L-NAME (10 μ M) 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 cavtratin. Ten, fifty, and seventy-five micromolars of cavtratin significantly increased IOP in WT mice, but did not significantly change IOP in NOS3 KO and DKO mice. Fifty micromolars of cavtratin significantly increased IOP in WT mice but lowers IOP in Cav1 KO mice ($P > 0.05$, $n = 6$). * $P < 0.05$, ** $P < 0.01$.

both 100 and 200 μ M L-NAME significantly increased IOP in WT, Cav1 KO, and NOS3 KO mice ($P < 0.05$, WT, 100 μ M, $n = 6$, 200 μ M, $n = 6$; Cav1, 100 μ M, $n = 6$, 200 μ M, $n = 6$; NOS3 KO, 100 μ M, $n = 4$, 200 μ M, $n = 6$). In WT mice, Δ IOP of 100 and 200 μ M 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, Δ IOP of 100 and 200 μ M 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,

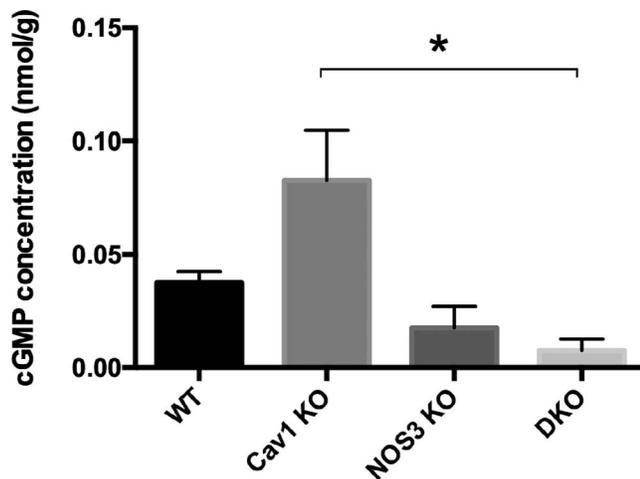


FIGURE 4. Comparison of cGMP concentration by ELISA between WT, Cav1 KO, NOS3 knockout (NOS3 KO), and NOS3 and Cav1 DKO mice. cGMP levels in DKO mice were compromised compared with Cav1 KO mice (mean \pm SD, $n = 4$, $*P < 0.05$). Data are analyzed by nonparametric Kruskal-Wallis ANOVA test.

Δ IOP of 100 and 200 μ 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 μ M) L-NAME increased IOP (from 12 to 14 mm Hg, Δ IOP = 2.4 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 μ 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).

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 μ M cavtratin in Cav1 KO mice (Fig. 3C; Supplementary Table S2). In DKO and NOS3 KO mice, cavtratin did not significantly change IOP (DKO: 10 μ M, $n = 6$; 50 μ M, $n = 6$; 75 μ M, $n = 6$; NOS3 KO: 10 μ M, $n = 6$; 50 μ M, $n = 6$; 75 μ M, $n = 5$; $P > 0.05$, Fig. 3C). Unexpectedly, 50 μ M cavtratin significantly increased IOP in WT mice (Δ IOP = 3.0 mm Hg) but lowered IOP in Cav1 KO mice (Δ IOP = 3.2 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.

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 NOS 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.³³ DKO mice exhibit normalized IOP, which is similar to WT mice (Fig. 2). 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 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 μ g SNP).³⁵ In monkeys, 50 and 500 μ 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 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.³⁸ 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 μ M cavtratin and an increase of IOP in response to 75 μ M cavtratin. The reason for this is unclear. We speculate that 50 μ M cavtratin may have inhibited eNOS overactivation related TM/SC outflow tissue damage^{39,40} 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,²⁶ 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.⁴³ 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 antagonists⁴⁷⁻⁴⁹ and prostaglandin analogs.⁵⁰⁻⁵² 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 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 NOS 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 (XYQ2013083), 211 Project of Fudan University (EHF158351), 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

1. Aga M, Bradley JM, Wanchu R, Yang YF, Acott TS, Keller KE. Differential effects of caveolin-1 and -2 knockdown on aqueous outflow and altered extracellular matrix turnover in caveolin-silenced trabecular meshwork cells. *Invest Ophthalmol Vis Sci.* 2014;55:5497-5509.
2. Herrnberger L, Ebner K, Junglas B, Tamm ER. The role of plasmalemma vesicle-associated protein (PLVAP) in endothelial cells of Schlemm's canal and ocular capillaries. *Exp Eye Res.* 2012;105:27-33.
3. Lei Y, Song M, Wu J, Xing C, Sun X. eNOS activity in CAV1 knockout mouse eyes. *Invest Ophthalmol Vis Sci.* 2016;57:2805-2813.
4. Lei Y, Zhang X, Song M, Wu J, Sun X. Aqueous humor outflow physiology in NOS3 knockout mice. *Invest Ophthalmol Vis Sci.* 2015;56:4891-4898.
5. Lei Y, Stamer WD, Wu J, Sun X. Endothelial nitric oxide synthase-related mechanotransduction changes in aged porcine angular aqueous plexus cells. *Invest Ophthalmol Vis Sci.* 2014;55:8402-8408.
6. Nathanson JA, McKee M. Alterations of ocular nitric oxide synthase in human glaucoma. *Invest Ophthalmol Vis Sci.* 1995;36:1774-1784.
7. Tamm ER. The trabecular meshwork outflow pathways: structural and functional aspects. *Exp Eye Res.* 2009;88:648-655.
8. Stamer WD, Lei Y, Boussommier-Calleja A, Overby DR, Ethier CR. eNOS, a pressure-dependent regulator of intraocular pressure. *Invest Ophthalmol Vis Sci.* 2011;52:9438-9444.
9. Kato T, Meguro A, Nomura E, et al. Association study of genetic variants on chromosome 7q31 with susceptibility to normal tension glaucoma in a Japanese population. *Eye (Lond).* 2013;27:979-983.
10. Abu-Amro KK, Kondkar AA, Mousa A, Osman EA, Al-Obeidan SA. Lack of association of SNP rs4236601 near CAV1 and CAV2 with POAG in a Saudi cohort. *Mol Vis.* 2012;18:1960-1965.
11. Kuehn MH, Wang K, Roos B, et al. Chromosome 7q31 POAG locus: ocular expression of caveolins and lack of association with POAG in a US cohort. *Mol Vis.* 2011;17:430-435.
12. Kang JH, Wiggs JL, Rosner BA, Haines J, Abdou W, Pasquale LR. Endothelial nitric oxide synthase gene variants and primary open-angle glaucoma: interactions with hypertension, alcohol intake, and cigarette smoking. *Arch Ophthalmol.* 2011;129:773-780.
13. Wiggs JL, Kang JH, Yaspan BL, et al. Common variants near CAV1 and CAV2 are associated with primary open-angle glaucoma in Caucasians from the USA. *Hum Mol Genet.* 2011;20:4707-4713.
14. Thorleifsson G, Walters GB, Hewitt AW, et al. Common variants near CAV1 and CAV2 are associated with primary open-angle glaucoma. *Nat Genet.* 2010;42:906-909.
15. Kang JH, Wiggs JL, Rosner BA, et al. Endothelial nitric oxide synthase gene variants and primary open-angle glaucoma: interactions with sex and postmenopausal hormone use. *Invest Ophthalmol Vis Sci.* 2010;51:971-979.
16. Henry E, Newby DE, Webb DJ, O'Brien C. Peripheral endothelial dysfunction in normal pressure glaucoma. *Invest Ophthalmol Vis Sci.* 1999;40:1710-1714.
17. Elliott MH, Ashpole NE, Gu X, et al. Caveolin-1 modulates intraocular pressure: implications for caveolae mechanoprotection in glaucoma. *Sci Rep.* 2016;6:37127.
18. Song M, Wu J, Lei Y, Sun X. Genetic deletion of the NOS3 gene in CAV1^{-/-} mice restores aqueous humor outflow function. *Invest Ophthalmol Vis Sci.* 2017;58:4976-4987.
19. Thompson MA, Prakash YS, Pabelick CM. The role of caveolae in the pathophysiology of lung diseases. *Expert Rev Respir Med.* 2014;8:111-122.
20. Fulton D, Gratton JP, Sessa WC. Post-translational control of endothelial nitric oxide synthase: why isn't calcium/calmodulin enough? *J Pharmacol Exp Ther.* 2001;299:818-824.
21. Bucci M, Gratton JP, Rudic RD, et al. In vivo delivery of the caveolin-1 scaffolding domain inhibits nitric oxide synthesis and reduces inflammation. *Nat Med.* 2000;6:1362-1367.

22. Chen Z, Bakhshi FR, Shajahan AN, et al. Nitric oxide-dependent Src activation and resultant caveolin-1 phosphorylation promote eNOS/caveolin-1 binding and eNOS inhibition. *Mol Biol Cell*. 2012;23:1388–1398.
23. Place AT, Chen Z, Bakhshi FR, Liu G, O'Bryan JP, Minshall RD. Cooperative role of caveolin-1 and C-terminal Src kinase binding protein in C-terminal Src kinase-mediated negative regulation of c-Src. *Mol Pharmacol*. 2011;80:665–672.
24. Bernatchez PN, Bauer PM, Yu J, Prendergast JS, He P, Sessa WC. Dissecting the molecular control of endothelial NO synthase by caveolin-1 using cell-permeable peptides. *Proc Natl Acad Sci U S A*. 2005;102:761–766.
25. Bakhshi FR, Mao M, Shajahan AN, et al. Nitrosation-dependent caveolin 1 phosphorylation, ubiquitination, and degradation and its association with idiopathic pulmonary arterial hypertension. *Pulm Circ*. 2013;3:816–830.
26. Zhao YY, Zhao YD, Mirza MK, et al. Persistent eNOS activation secondary to caveolin-1 deficiency induces pulmonary hypertension in mice and humans through PKG nitration. *J Clin Invest*. 2009;119:2009–2018.
27. Mao M, Sudhahar V, Ansenberger-Fricano K, et al. Nitroglycerin drives endothelial nitric oxide synthase activation via the phosphatidylinositol 3-kinase/protein kinase B pathway. *Free Radic Biol Med*. 2012;52:427–435.
28. Fernandez-Durango R, Fernandez-Martinez A, Garcia-Feijoo J, et al. Expression of nitrotyrosine and oxidative consequences in the trabecular meshwork of patients with primary open-angle glaucoma. *Invest Ophthalmol Vis Sci*. 2008;49:2506–2511.
29. Ebner A, Kurbis N, Brandt A, et al. Endothelial nitric oxide synthase-induced hypertrophy and vascular dysfunction contribute to the left ventricular dysfunction in caveolin-1^{-/-} mice. *Can J Cardiol*. 2017;33:1716–1724.
30. Mierke J, Christoph M, Pfluecke C, et al. Atheroprotective role of Caveolin-1 and eNOS in an innovative transplantation model is mainly mediated by local effects. *Biochim Biophys Acta*. 2017;1863:529–536.
31. Parolini I, Sargiacomo M, Galbiati F, et al. Expression of caveolin-1 is required for the transport of caveolin-2 to the plasma membrane. Retention of caveolin-2 at the level of the golgi complex. *J Biol Chem*. 1999;274:25718–25725.
32. Yu J, Bergaya S, Murata T, et al. Direct evidence for the role of caveolin-1 and caveolae in mechanotransduction and remodeling of blood vessels. *J Clin Invest*. 2006;116:1284–1291.
33. Murata T, Lin MI, Huang Y, et al. Reexpression of caveolin-1 in endothelium rescues the vascular, cardiac, and pulmonary defects in global caveolin-1 knockout mice. *J Exp Med*. 2007;204:2373–2382.
34. Schneemann A, Leusink-Muis A, van den Berg T, Hoyng PE, Kamphuis W. Elevation of nitric oxide production in human trabecular meshwork by increased pressure. *Graefes Arch Clin Exp Ophthalmol*. 2003;41:321–326.
35. Nathanson JA. Nitrovasodilators as a new class of ocular hypotensive agents. *J Pharmacol Exp Ther*. 1992;260:956–965.
36. Heyne GW, Kiland JA, Kaufman PL, Gabelt BT. Effect of nitric oxide on anterior segment physiology in monkeys. *Invest Ophthalmol Vis Sci*. 2013;54:5103–5110.
37. Giuffrida S, Bucolo C, Drago F. Topical application of a nitric oxide synthase inhibitor reduces intraocular pressure in rabbits with experimental glaucoma. *J Ocul Pharmacol Ther*. 2003;19:527–534.
38. Chang JY, Stamer WD, Bertrand J, et al. Role of nitric oxide in murine conventional outflow physiology. *Am J Physiol Cell Physiol*. 2015;309:C205–C214.
39. Lei Y, Song M, Wu J, Xing C, Sun X. eNOS activity in CAV1 knockout mouse eyes. *Invest Ophthalmol Vis Sci*. 2016;57:2805–2813.
40. Zhao YY, Zhao YD, Mirza MK, et al. Persistent eNOS activation secondary to caveolin-1 deficiency induces pulmonary hypertension in mice and humans through PKG nitration. *J Clin Invest*. 2009;119:2009–2018.
41. Bucolo C, Drago F. Carbon monoxide and the eye: Implications for glaucoma therapy. *Pharmacol Ther*. 2011;130:191–201.
42. Drago F, Bucolo C. Therapeutic potential of nitric oxide modulation in ocular diseases. *Drug News Perspect*. 2010;23:430–437.
43. Pong WW, Eldred WD. Interactions of the gaseous neuro-modulators nitric oxide, carbon monoxide, and hydrogen sulfide in the salamander retina. *J Neurosci Res*. 2009;87:2356–2364.
44. Wang L, McLeod H, Weinshilboum R. Genomic medicine genomics and drug response. *N Engl J Med*. 2011;364:1144–1153.
45. Roden DM, George AL. The genetic basis of variability in drug responses. *Nat Rev Drug Discov*. 2002;1:37–44.
46. Hauser AS, Chavali S, Masuho I, et al. Pharmacogenomics of GPCR drug targets. *Cell*. 2018;172:41–54.e19.
47. McCarty CA, Burmester JK, Mukesh BN, Patchett RB, Wilke RA. Intraocular pressure response to topical beta-blockers associated with an ADRB2 single-nucleotide polymorphism. *Arch Ophthalmol*. 2008;126:959–963.
48. Nieminen T, Uusitalo H, Mäenpää J, et al. Polymorphisms of genes CYP2D6, ADRB1 and GNAS1 in pharmacokinetics and systemic effects of ophthalmic timolol. A pilot study. *Eur J Clin Pharmacol*. 2005;61:811–819.
49. Inagaki Y, Mashima Y, Fuse N, et al. Polymorphism of beta-adrenergic receptors and susceptibility to open-angle glaucoma. *Mol Vis*. 2006;12:673–680.
50. Cui XJ, Zhao AG, Wang XL. Correlations of AFAP1, GMDS and PTGFR gene polymorphisms with intra-ocular pressure response to latanoprost in patients with primary open-angle glaucoma. *J Clin Pharm Ther*. 2017;42:87–92.
51. Sakurai M, Higashide T, Ohkubo S, Takeda H, Sugiyama K. Association between genetic polymorphisms of the prostaglandin F2 α receptor gene, and response to latanoprost in patients with glaucoma and ocular hypertension. *Br J Ophthalmol*. 2014;98:469–473.
52. Ussa F, Fernandez I, Brion M, et al. Association between SNPs of metalloproteinases and prostaglandin F2 α receptor genes and latanoprost response in open-angle glaucoma. *Ophthalmology*. 2015;122:1040–1048.e1044.
53. Emam WA, Zidan HE, Abdulhalim BE, Dabour SA, Ghali MA, Kamal AT. Endothelial nitric oxide synthase polymorphisms and susceptibility to high-tension primary open-angle glaucoma in an Egyptian cohort. *Mol Vis*. 2014;20:804–811.
54. Magalhães da Silva T, Rocha AV, Lacchini R, et al. Association of polymorphisms of endothelial nitric oxide synthase (eNOS) gene with the risk of primary open angle glaucoma in a Brazilian population. *Gene*. 2012;502:142–146.
55. Ayub H, Khan MI, Micheal S, et al. Association of eNOS and HSP70 gene polymorphisms with glaucoma in Pakistani cohorts. *Mol Vis*. 2010;16:18–25.
56. Xiang Y, Dong Y, Li X, Tang X. Association of common variants in eNOS gene with primary open angle glaucoma: a meta-analysis. *J Ophthalmol*. 2016;2016:1348347.