

Decreased Intraocular Pressure Induced by Nitric Oxide Donors Is Correlated to Nitrite Production in the Rabbit Eye

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Purpose. To evaluate the effect of intraocular administration of nitric oxide (NO) donors in the rabbit eye on intraocular pressure (IOP), inflammation, and toxicity.

Methods. Intravitreal and intracameral injections of two NO donors, SIN-1 and SNAP, and SIN-1C and BSS were performed. Clinical examination, IOP measurements, protein evaluation in aqueous humor, and histologic analysis of the ocular globes were realized. Nitric oxide release was demonstrated by nitrite production in the aqueous humor and in the vitreous using the Griess reaction.

Results. The drastic decrease of IOP, observed after a single NO donor injection, was correlated directly with nitrite production and, thus, to NO release. Injection of inactive metabolite of SIN-1, SIN-1C, which is not able to release NO, did not modulate IOP. When administered in the aqueous humor or in the vitreous, NO did not diffuse from one segment of the eye to another. No inflammation or histologic damage was observed as a result of a single NO donor administration.

Conclusions. Nitric oxide is implicated directly in the regulation of IOP and its acute, and massive release into the rabbit eye did not induce inflammation or other growth toxic effects on the ocular tissues. Invest Ophthalmol Vis Sci. 1996;37:1711-1715.

Glaucoma seems to be a multifactorial condition with field loss that may lead to blindness. It is unclear whether the primary event is neurogenic or vascular or is the result of abnormal regulation of the intraocular pressure (IOP). However, IOP consistently has been found to be one of the most important risk factors in glaucomatous injury, and it has been demonstrated that lowering the IOP would be protective.^{1,2} The role

of nitrovasodilators was suspected because their systemic administration induces a decrease of the intraocular pressure without altering systemic blood pressure.³ More recently, Nathanson demonstrated that topical administration of nitrovasodilators lowers IOP⁴ and that this change was caused by the decrease of the resistance of the aqueous humor leaving the eye and not to a reduction of aqueous humor production.⁵ The effects of these compounds, identified as nitric oxide (NO) donors, suggest the role of NO in the regulation of IOP. The enzyme responsible for NO formation, the NO synthase (NOS), has been identified in the rat eye. It was found essentially in peripheral ocular nerve fibers arising from parasympathetic ganglia.⁶ A recent report demonstrated that in humans, another isoform of NOS is expressed in Schlemm's canal, in the trabecular meshwork, and in the ciliary muscle.⁷ On the other hand, topical application of nitroglycerin has an inconsistent effect on IOP. The nitroglycerin-induced IOP decrease in monkeys obtained by Schuman et al⁵ in normal eyes, has not been reproduced either in normal glaucoma or in a model of laser-induced glaucoma.⁸ However, the pathogenesis of the laser glaucoma model might be different from open angle glaucoma in humans, which explains the lack of efficiency of nitroglycerin. Because the effect of NO donors is controversial, we investigated the effect on IOP of two NO donors administered by injection into the ocular media and the subsequent release of nitrite, which reflects NO production. The potential inflammatory and toxic effects of these compounds were evaluated by protein infiltration and histologic analysis.

MATERIAL AND METHODS. Animals. The rabbits used in this study, Fauve de Bourgogne pigmented females (weight range, 2.5 to 3 kg; age range, 10 to 12 weeks) were cared for and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Only one eye was treated for each animal.

Reagents. Sydnonimide analogs, 3 morpholino-sydnonimide (SIN-1), N-morpholinoimino-acetonyl (SIN-1C), and S-nitrosoacetylpenillamine (SNAP), were obtained from TEBU (Biomol Research Laboratories, Plymouth, MA). Balanced saline solution (BSS) was obtained from Alcon (Rueil-Malmaison, France). Reagents were diluted in the BSS solvent and injected as soon as dissolved.

Measure of Intraocular Pressure. Rabbits were housed under standard conditions at least 8 days before the experiments. They were exposed to natural illumination. Experiments were scheduled for the same time each day, from 9 AM until 6 PM, and were performed

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Supported by a grant from the Fondation de France (Louis Fouassier) and by IPSEN Laboratories.

Proprietary interest category: N.

Submitted for publication October 31, 1995; revised January 16, 1996; accepted March 28, 1996.

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during winter. Intraocular pressure was determined by using both Schiøtz and Goldmann tonometer (Haag-Sreit, Bern, Switzerland). Before each set of measures, one drop of oxybuprocaine chlorhydrate 0.4% (Novesine, Chibret, France) was instilled three times every 10 minutes. Five measures of IOP were performed to accustom the rabbit to the procedure before experiments were begun. At each time, the mean of three measures was calculated for the Schiøtz and the Goldmann measures. Finally, the mean of these measures was calculated.

Intraocular Injections. Nitric oxide donors were administered by direct intraocular injections into the vitreous (V) or the aqueous humor (AH) using a Hamilton syringe (Poly-Labo, Paris, France) and 30 g needles (Microlance 3, Becton Dickinson, Spain). Intravitreal injections were performed through the sclera, 3 mm from the limbus in the temporal and superior quadrant. Intracameral injections were performed through the cornea 2 mm from the limbus to avoid limbus vessels. Fifty microliters was injected to reduce IOP variations from injections.

Nitrite Evaluation. Aqueous humor was obtained by anterior chamber puncture (1 ml syringe and 30 g needle). Vitreous samples were obtained from an anterior vitrectomy. After the last IOP measurement, animals were sedated with 2 mg/kg midazolam (Hypnovel; Roche, Neuilly-sur-Seine, France) given intramuscularly and were anesthetized with 60 mg/kg intramuscular ketamine (Ketalar, Parke-Davis, Courbevoie, France). First an extracapsular extraction of the lens was performed, then the posterior capsule and the posterior hyaloid membranes were dissected to perform the vitrectomy. This technique was used to prevent any bleeding because of a two-port vitrectomy, which can interfere with nitrite dosage. Fifty microliters of AH or V were assayed in triplicate using the spectrophotometric method based on the Griess reaction, as previously described.⁹

Clinical Evaluation. Animals were examined using a Haag-Steit 900 (Bern, Switzerland) biomicroscope before injection, after injection, and before every IOP measurement, and then were photographed. Anterior and posterior segments of the eye were examined as well as the retina by using a 90 D Volk II lens.

Protein Evaluation. Protein was assayed in AH samples according to the method of Bradford (Bio Rad kit, Les Ulis, France). For the quantification of protein, bovine serum albumin was used as a standard. Aqueous humor samples were obtained by anterior puncture 1 or 4 hours after SIN-1 or SNAP injection.

Histologic Analysis. Animals were killed with lethal doses of intramuscular pentobarbital (Nembutal; Abbot, Saint-Remy sur Avre, France). Light microscopy analysis of ocular globes was performed 1 hour, 24 hours, 15 days, and 30 days after NO donor treatment.

Statistical Analysis. Results are expressed as means \pm standard deviation and were analyzed by the nonpara-

metric Mann-Whitney test. $P < 0.05$ was considered significant.

RESULTS. Intraocular Pressure Decrease. The basal IOP in pigmented Fauve de Bourgogne rabbits was 21 ± 3.5 mm Hg measured by Schiøtz tonometer and 23 ± 2 mm Hg measured by Goldmann tonometer (unpaired *t*-test, $P = 0.08$, $n = 8$), which is slightly higher than the IOP reported by others on New Zealand White rabbits.¹⁰ Intravitreal injection of 50 μ l SIN-1 (20 mM) induced a transitory increase of IOP just after injection ($33\% \pm 3\%$), which also was observed with SIN-1C and BSS (Fig. 1). This increase was caused by the injected volume in the vitreous cavity and was not the result of a specific action of NO donors. Normal values of IOP were observed approximately 30 minutes after the injection. In SIN-1-injected eyes, a mean decrease in IOP of $71\% \pm 5\%$ was observed 60 minutes after injection. Normal values were recovered approximately 3 hours later (Fig. 1). No effect on IOP was observed as a result of intravitreal injection of the inactive metabolite of SIN-1, SIN-1C¹¹, or BSS (Fig. 1).

The intracameral injection of 50 μ l SIN-1 (20 mM) initially induced a $44\% \pm 6\%$ ($n = 4$) decrease in the basal IOP resulting from the intracameral puncture because the same lowering was observed in the BSS-injected group ($49\% \pm 4\%$, $n = 4$). Figure 2 shows in the SIN-1-treated group, but not in the BSS-treated group, a decrease of $68\% \pm 8\%$ ($n = 6$) in IOP approximately 1 hour after injection. Normal IOP was recovered approximately 3 hours after injection. This change in the IOP was not observed in the BSS-injected group nor in the SIN-1C-treated group (Fig. 2).

The intravitreal injection of 50 μ l SNAP (20

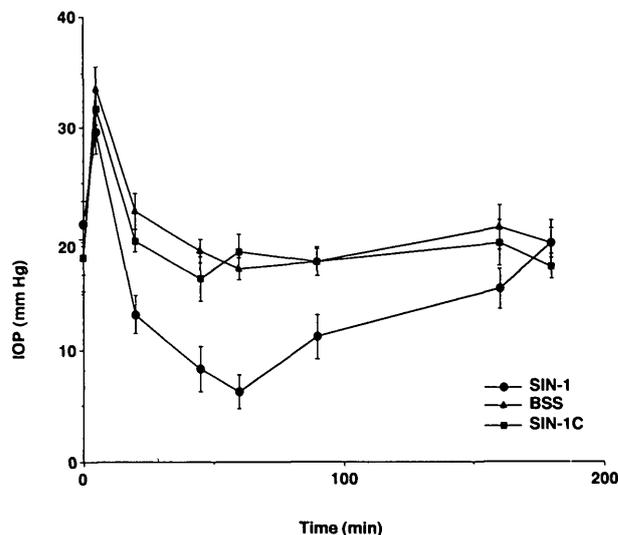


FIGURE 1. Measurements of IOP after intravitreal injection of 50 μ l SIN-1 and SIN-1C (20 mM). Values are means \pm SD.

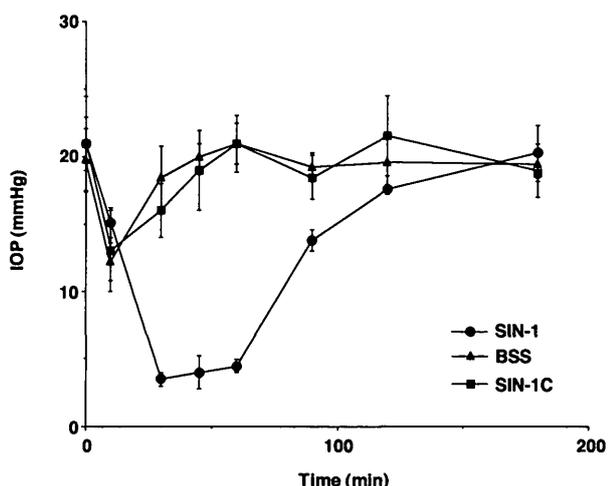
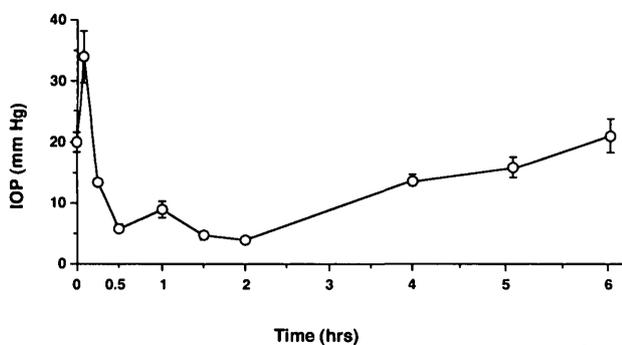
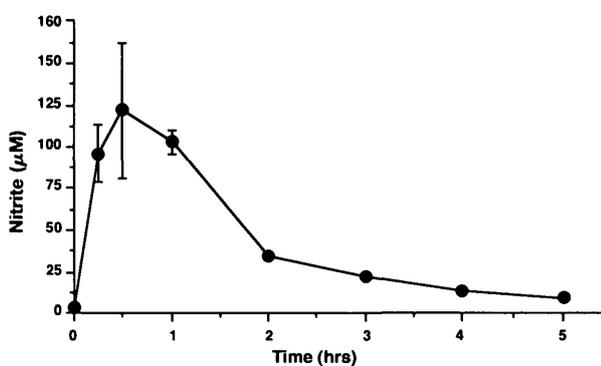


FIGURE 2. Measurement of intraocular pressure after intracameral injection of 50 μ l SIN-1 and SIN-1C (20 mM). Values are means \pm SD.



A

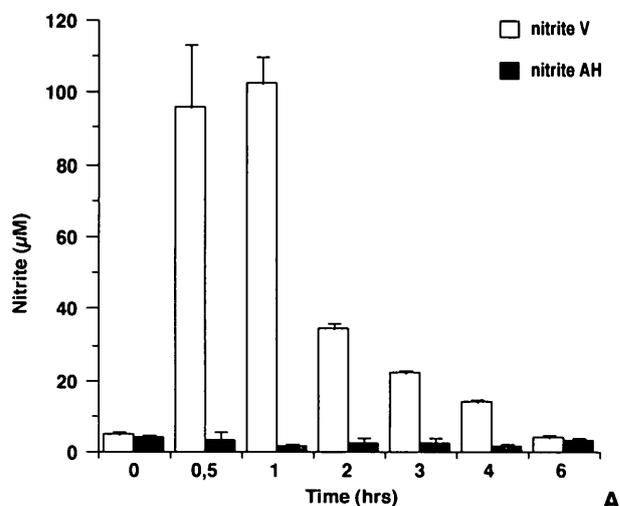


B

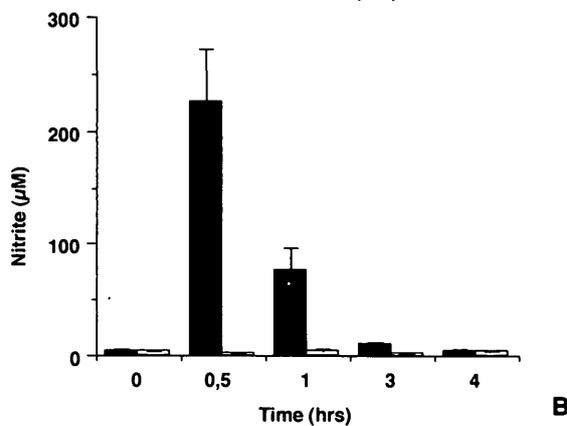
FIGURE 3. Correlation between intraocular pressure and nitrite production after intravitreal injection of SNAP. After injection of 50 μ l SNAP (20 mM), the intraocular pressure was measured 15, 30, and 60 minutes and every hour until return to normal values. At each time point, four rabbits underwent vitrectomy to sample the vitreous and were, therefore, excluded from the time course of intraocular pressure determination. Values are given as means \pm SD.

mM) initially induced an increase of 30% in the IOP caused by the volume injected into the vitreous cavity (Fig. 3A). As described above, this variation in IOP also was observed in the control BSS-injected eyes and was not caused by NO donors. The normal IOP recurred after 15 to 30 minutes (23 ± 8 minutes), followed by a dramatic decrease of 72% in the IOP was measured 90 minutes after SNAP injection. Normal values were recovered approximately 6 hours after injection (Fig. 3A). In experiments not reported herein, intracameral injection of SNAP induced changes in IOP similar to those observed with SIN-1, except that IOP decrease lasted 6 hours with the latter.

Evaluation of Nitrite Production in Aqueous Humor and Vitreous. When SNAP was injected into the vitreous, nitrite production was augmented markedly in the V after 30 minutes and lasted 6 hours after injection (Figs. 3B, 4A). The time course and amount of nitrite release was correlated directly to IOP low-



A



B

FIGURE 4. Evaluation of nitrites in aqueous humor and vitreous after either intravitreal (A) or intracameral (B) administration of 50 μ l SNAP (20 mM). At each time point, aqueous humor was first sampled before performing an anterior vitrectomy to sample the vitreous. Values are given as means \pm SD.

ering (Fig. 3). No nitrite increase was detected in the AH after intravitreal injection of SNAP (Fig. 4A). Identical correlations have been obtained with intravitreal injection of SIN-1 (data not shown). SIN-1C, which is unable to release NO, did not produce nitrite in the V 1 hour after injection ($3.1 \pm 2.7 \mu\text{M}$, $n = 4$).

After intracameral injection of SNAP, a maximal amount of nitrite was detected in the AH after 30 minutes and slowly decreased to the control values within 4 hours (Fig. 4B). No nitrites were detected in the V after intracameral injection of SNAP, suggesting that NO donor administration in either the anterior or the posterior segment of the eye did not diffuse from one segment to the other.

Protein Evaluation. The protein concentration in AH, 1 hour and 4 hours after 50 μl SIN-1 (20 mM) intravitreal injection, were, respectively, 0.4 ± 0.21 and 0.45 ± 0.24 mg/ml ($n = 4$), compared, respectively, to 0.42 ± 0.09 and 0.43 ± 0.25 mg/ml ($n = 4$) in the BSS-injected group ($P = 0.7$, $P = 0.68$). In the group of rabbits injected with 50 μl SNAP (20 mM), the protein concentration in AH were 0.55 ± 0.04 and 0.36 ± 0.4 mg/ml ($P = 0.29$, $P = 0.28$; $n = 4$), respectively, at 1 hour and 4 hours after the injection. No statistical difference in the concentration of protein in the group of rabbits injected with NO donors, compared to the group of rabbits injected with BSS, was observed.

Histologic Analysis. Histologic analysis of the eyes 1 hour, 24 hours, 15 days, and 30 days after treatment showed no lesions in the corneal endothelium, the iris, or the ciliary body, and no damage in the retina. No signs of inflammation, such as cell infiltration, was observed, and no cell suffering or death were detected as a consequence of the administration of the high concentrations of NO donors (data not shown).

DISCUSSION. Direct injections of NO donors into the ocular globe induced a drastic lowering of the IOP, more marked compared to topical application of these compounds.⁴ We demonstrate, for the first time, that there is a direct production of NO in the ocular media by nitrite evaluation in the AH and in the V after NO donor injection and that the IOP decrease is correlated to NO production. A similar time-course of SIN-1-induced IOP decrease was obtained when the NO donor was injected into the V or into the AH. The NO produced either in the AH or in the V might reach the ciliary muscle directly to interact with guanylate cyclase, leading to a cyclic guanosine monophosphate increase, responsible for the IOP modulation.^{4,12} Furthermore, injection of the inactive product, SIN-1C, did not release NO and then did not decrease IOP, confirming the direct role of NO on the regulation of IOP. In agreement with our data, topical application of the inactive precursor of SIN-1, molsidomine,

failed to decrease IOP in the rabbit.⁴ SNAP action on IOP lasted longer than SIN-1 after either intravitreal or intracameral injection, probably because of the differential kinetics of NO release by the two compounds. Nitric oxide donors and the NO released do not seem to diffuse from V to AH and from AH to V because no increase in nitrite is observed in AH after intravitreal injection nor in V after intracameral injection. High concentrations of thiols located in the lens might act as scavengers for NO and serve as a barrier to the diffusion of NO from one segment of the eye to the other. Moreover, diffusion from the AH into the V or to the V into the anterior aqueous might be diverted by the increased outflow induced by NO donors.^{4,5} As a result of high concentrated NO solutions in the globe, a transitory vasodilation of conjunctival and episcleral vessels was observed that lasted approximately 20 minutes. No flare in the AH or in the V and no exudation of protein or cell infiltration were observed after NO administration, as demonstrated by protein concentration in AH and histologic analysis of whole ocular globes. These results suggest that a single injection of NO donors failed to induce a breakdown of the blood–aqueous barrier, even at the maximal decrease of IOP. Thus, no relation might be established between IOP changes and a breakdown of the blood–aqueous barrier. Furthermore, the observation of the lenses 1 month after acute production of NO in the vitreous did not show any cataractogenic effect of these compounds. Recent studies have demonstrated that NO can be involved in experimentally induced inflammatory responses, such as endotoxin-induced uveitis, which could partially be inhibited by intraperitoneal administration of a NOS inhibitor.⁹ However, acute excessive NO might not induce inflammation directly or cause tissue damage, as shown by our histologic analysis of ocular globe at different times after one NO donor injection. The implication of NO in the inflammatory response or in retinal toxicity could result either from chronic effects of NO or from a combination with other free radicals or cytokines produced during the inflammatory process.

In conclusion, these experiments demonstrated the direct correlation between intraocular NO production and IOP lowering and the absence of a toxic effect by the massive administration of NO donors. Currently, we are investigating whether chronic administration of NO could lead to tissue damage or inflammation.

Key Words

intraocular pressure, nitric oxide, rabbit, toxicity

References

1. Kolker AE. Visual prognosis in advanced glaucoma: A comparison of medical and surgical therapy for reten-

- tion of vision in 101 eyes with advanced glaucoma. *Trans Am Ophthalmol Soc.* 1977;75:539–545.
2. Werner EB, Drance SM, Schulzer M. Trabeculectomy and the progression of glaucomatous visual field loss. *Arch Ophthalmol.* 1983;95:1374–1377.
 3. Wiseman A, Wiseman V. Untersuchungen zur ambulanten und perioperativen Augeninnendrucksenkung mit organischen Nitraten. *Klin Monatsbl Augenheilkd.* 1980;177:292–295.
 4. Nathanson JA. Nitrovasodilators as a new class of ocular hypotensive agents. *J Pharmacol Exp Ther.* 1992;260:956–965.
 5. Schuman J, Erickson K, Nathanson JA. Nitrovasodilator effects on intraocular pressure and outflow facility in monkeys. *Exp Eye Res.* 1994;58:99–105.
 6. Yamamoto R, Brecht DS, Snyder SH, Stone RA. The localization of nitric oxide synthase in the rat eye and related cranial ganglia. *Neuroscience.* 1993;54:189–200.
 7. Nathanson JA, McKee M. Identification of an extensive system of nitric oxide producing cells in the ciliary muscle and outflow pathway of the human eye. *Invest Ophthalmol Vis Sci.* 1995;36:1765–1773.
 8. Wang RF, Podos SM. Effect of the topical application of Nitroglycerin on intraocular pressure in normal and glaucomatous monkeys. *Exp Eye Res.* 1995;60:337–339.
 9. Goureau O, Bellot J, Thillaye B, Courtois Y, de Kozak Y. Increased nitric oxide production in endotoxin-induced uveitis. *J Immunol.* 1995;22:6518–6523.
 10. Bar-Ilan A. Diurnal and seasonal variations in intraocular pressure in the rabbit. *Exp Eye Res.* 1984;39:175–181.
 11. Noack E, Feilish M. Molecular aspects underlying the vasodilators action of molsidomine. *J Cardiovasc Pharmacol.* 1989;14(suppl 11):S1–S5.
 12. Becker B. Topical 8-bromo-cyclic GMP lowers intraocular pressure in rabbits. *Invest Ophthalmol Vis Sci.* 1990;31:1647–1649.