Enhanced Oxygen Saturation in Optic Nerve Head of Non-Human Primate Eyes Following the Intravitreal Injection of NCX 434, an Innovative Nitric Oxide-Donating Glucocorticoid

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Abstract

Purpose: Hypoxia of the retina and optic nerve head (ONH) is believed to be pivotal in the development of ocular vascular disorders, including diabetic macular edema (DME). Glucocorticoids are among the most effective agents for the treatment of back of the eye diseases. However, this class of compounds is highly liable to increase intraocular pressure (IOP) and does not improve ocular perfusion or tissue oxygenation. Nitric oxide (NO) has vasodilating properties and lowers IOP in experimental models and humans, suggesting that its properties might complement those of glucocorticoids. NCX 434 is an NO-donating triamcinolone acetonide (TA) that is less likely to increase IOP while targeting both the vascular and inflammatory components of DME.

Methods: NCX 434 was studied *in vitro* with respect to its NO-releasing properties in isolated methoxamine-precontracted rabbit aortic rings and glucocorticoid-like activity in recombinant human glucocorticoid receptors. IOP and oxygen saturation in the ONH and overlaying arteries and veins were studied in the anesthetized cynomolgus monkey. Measurements were taken using, respectively, an applanation tonometer and a hyper-spectral imaging system before and 7, 14, 21, 31 and 41 days after the intravitreal injection of NCX 434 (5.8 mg/eye) or TA equimolar doses (4.0 mg/eye).

Results: NCX 434 inhibited ³H-dexamethasone-specific binding ($IC_{50}=34\pm5$ nM) on human glucocorticoid receptors and elicited NO-dependent aortic ring relaxation (EC_{50} of 0.5 ± 0.1 μ M, E_{max} 98.9%). In monkey eyes, NCX 434 enhanced, whereas TA did not, oxygen saturation in various ONH areas (* $P<0.05$ vs. basal), decreased it in veins, and did not affect it in the overlaying arteries. Neither NCX 434 nor TA altered IOP significantly at all time points. However, at 31 days post-treatment TA appeared to start increasing IOP ($\Delta IOP = +3.31\pm0.51$ mmHg, 30.8%, over baseline, NS).

Conclusions: NCX 434 enhances ocular tissue oxygenation. This feature appears to depend on its NO-donating properties; thus, the compound deserves to be further investigated for the treatment of DME and other ocular disorders with impaired ocular perfusion.

Introduction

THE RETINA REQUIRES a constant supply of oxygen, which is regulated primarily by the choroidal blood supply.¹⁻³ Serious pathological conditions arise when this supply is either excessive or deficient. For example, retinal neovascularization and macular edema, the major hallmarks of proliferative diabetic retinopathy, are thought to partially result

from faulty vascular autoregulation and hypoxia,^{4,5} whereas supplemental oxygen to the optic nerve head (ONH) ameliorates diabetic macular edema (DME) symptoms.⁶

There are no approved drugs for the treatment of DME to date. While anti-VEGF agents have successfully been tested in clinical trials with positive outcome,⁷ glucocorticoids (eg, triamcinolone acetonide [TA], Kenalog[®]) still remain among the most used off-label drug for the treatment of this

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disorder. Glucocorticoids effectively decrease the edema and inflammation when administered intravitreally to DME patients.⁸ Despite that, the use of this class of compounds has been reported to increase the incidence of hypertensive glaucoma and cataract^{9–11} and their effect on ocular micro- and macro-vasculature remains controversial. Further, glucocorticoids, by increasing intraocular pressure (IOP), might further reduce oxygen availability to critical structures in the eye.^{12,13}

Nitric oxide (NO) is produced by 3 distinct isoforms of nitric oxide synthase (NOS). Two of these are constitutive enzymes (neuronal NOS or NOS-I and endothelial NOS or NOS-III) requiring intracellular Ca^{++} accumulation and are expressed in many cell types within the ocular compartment,¹⁴ whereas the third isoform (NOS-II or iNOS) is mainly expressed in immune cells, is induced in response to pro-inflammatory stimuli, and produces large amounts of NO in a Ca^{++} -independent manner.^{14,15}

It has been reported that a low physiological amount of NO activates soluble guanylyl cyclase and vascular relaxation,^{15,16} favors neuronal transmission, and modulates gene transcription through nuclear factor NF-kappa B signaling.^{17,18} Conversely, large quantities are thought to result in abnormal neuronal activity and central sensitization,¹⁹ activate proinflammatory cytokines, and promote tissue-specific oxidative and nitrosative stress.

In ocular compartments, NO has been shown to regulate choroidal blood flow,^{19,20} ophthalmic artery perfusion,²¹ and IOP^{22,23} likely by modulating aqueous humor production and drainage through the trabecular meshwork and Schlemm's canal, suggesting that NO-mediated effects might complement those of glucocorticoids. Consistent with that, a recent report²⁴ has shown that the NO-donating dexamethasone, NCX 1021, is devoid of IOP-raising effects and increases ocular blood perfusion to rabbit eyes. This study, however, did not address whether NO supply is also accompanied with an enhanced oxygen delivery to ocular tissues.

Spectral imaging offers a means of evaluating the oxygenation status of the retina, in both human and animal studies. More specifically, a noninvasive hyperspectral imaging technique, in conjunction with specialized algorithms to compute blood saturation values, has been shown to reliably map relative values of oxygen saturation in the retina and optic disc under physiological conditions^{25,26} and in response to elevated IOP or oxygen breathing in a non-human primate model.¹³

Here, using a hyperspectral imaging system, we have compared the effect of NCX 434 an NO-donating TA to that of the reference TA on oxygen saturation of the ONH and overlying arteries and veins in the cynomolgus monkey model. Concomitantly, we also monitored the effects of these treatments on IOP.

The results indicate that NCX 434 does not increase IOP, but it enhances oxygen saturation in the ONH structures.

Methods

Chemical structure, synthesis, and physicochemical properties

NCX 434 [2-((8S,9R,10S,11S,13S,14S,16S,17R)-9-fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl 2-(6-(nitrooxy)hexanamido)acetate] was synthesized according to standard procedures as shown in the scheme reported in Fig. 1. In brief, to a stirred solution of TA (2 g, 4.6 mmol) in acetone (130 mL), (BOC-Gly, 1.29 g, 7.36 mmol), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDAC, 1.41 g, 7.36 mmol), and 4-(dimethylamino)pyridine (DMAP, 0.46 g, 0.46) were added and the solution stirred overnight at room temperature. The reaction was monitored by TLC (n-hexane/ethyl acetate 1/1), the solvent evaporated, and the compound purified by chromatography (Biotage 100 SNAP), 1 column volume at 50% ethyl acetate, from 50% to 100% ethyl acetate in 8 column

FIG. 1. Scheme of the synthetic pathway for NCX 434. Schematic representation of the synthetic pathway used to synthesize NCX 434.

volumes, 1 column volume at 100% ethyl acetate to give 2.37 g of compound 1. Compound 1 (2.37 g 4 mmol) was dissolved in 100 mL of dichloromethane and HCl was bubbled through the solution. After 2 h, the solvent was evaporated, giving 2.1 g of compound 2. To a stirred suspension of compound 2 (2.1 g, 4 mmol) in dichloromethane (100 mL), compound 3 (1.374 g, 4 mmol) and DMAP (0.733 g, 6 mmol) were added and the reaction stirred at room temperature for 24 h. The reaction was monitored by TLC (n-hexane/ethyl acetate 3/7), the solvent evaporated, and the compound purified by chromatography (Biotage 100 SNAP), 1 column volume at 50% ethyl acetate, from 50% to 100% ethyl acetate in 10 column volumes, 2 column volumes at 100% ethyl acetate to give 1.3 g of NCX 434. NCX 434 is a white powder with a melting point of 184°C and a purity index of >95.0% determined by NMR spectroscopy (Bruker Advance 300 MHz in DMSO- d_6) and Waters Acquity UPLC System, column: Acquity UPLC BEH C_{18} 50×2.1 mm (1.7 μ m), eluent: water + 0.1% formic acid/acetone/nitrile + formic acid 0.1% from 90%/10% to 0%/100% in 3 min, 0%/100% for 1.5 min and to 90%/10% in 0.1 min, retention time = 2.03 min.

In vitro studies

Glucocorticoid receptor binding assay. Recombinant human glucocorticoid receptors (hGR) expressed in Sf9 insect cells were used as a test system (Invitrogen). The assay was performed by adding to incubation buffer (KEMD: 10 mM potassium phosphate, pH 7.4, containing 0.1 mM EDTA, 5 mM DTT, 10 mM Na_2MoO_4), either cold dexamethasone (final concentration 10 μ M, for nonspecific binding), vehicle (total binding), or test compounds at the desired concentration. 3 [H]-dexamethasone was used as a radiolabeled ligand (specific activity = 88 Ci/mmol, GE Healthcare) to reach the final concentration of 20 nM.

The reaction was started by the addition of recombinant hGR (specific activity: 15.4 pmol/mg protein) at the final concentration of 4 fmol/ μ L. The assay plate was then incubated under shaking at equilibrium on ice for 2 h. At the end of the incubation time, 50 μ L of 5% charcoal (w/v; Sigma-Aldrich) diluted in KEMD was used to scavenge free 3 [H]-dexamethasone. Samples were finally transferred in a glass-fiber filter plate (pore dimension: 1 μ m) and filtered under vacuum, and the supernatants collected and evaporated overnight at room temperature. The day after, 150 μ L/well of scintillation cocktail (Supermix; Perkin Elmer) was added to each well and later counted in a Microbeta instrument.

Vascular relaxing activity in methoxamine-precontracted rabbit aortic rings

In these experiments, animals were cared for and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Experiments were performed in accordance with protocols approved by the Animal Care Committees and all efforts were made to limit the number of animals and to minimize animal suffering. Male New Zealand White rabbits (1.8–2 kg) were used. The animals were anesthetized with sodium thiopental (50 mg/kg, iv), sacrificed by exsanguination, and the aorta dissected. The aortas were then placed in Krebs-HEPES buffer (pH 7.4; composition mM: NaCl 130.0, KCl 3.7, $NaHCO_3$ 14.9, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, Glucose 11.0, HEPES 10.0,

$CaCl_2 \cdot 2H_2O$ 1.6). Connective tissue was removed and aortas were cut into ring segments (4–5 mm in length). Each ring was placed in a 5 mL tissue bath filled with Krebs-HEPES buffer (37°C) aerated with 95% O_2 and 5% CO_2 and attached to a force transducer (Grass FT03), connected to a BIOPAC MP150 System for measurement of the isometric tension.

Preparations were allowed to equilibrate for 1 h at a resting tension of 2 g with changes of the buffer every 15 min and precontracted submaximally with methoxamine (3 μ M), and then a cumulative concentration–response curve to the compounds (0.01–100 μ M) was obtained. The time intervals between doses were based on the time needed to reach a full response. The results are given as mean $\pm$ SEM. The responses are expressed as percentage relaxation and plotted versus concentration.

Studies in cynomolgus monkeys

The use of animals in this study was approved by the LSU Health Sciences Center Institutional Animal Care and Use Committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Five cynomolgus monkeys with normal eyes were used. For a hyperspectral imaging session, monkeys were anesthetized with intramuscular ketamine (7–10 mg/kg) and xylazine (0.6–1 mg/kg) and intravenous pentobarbital (25–30 mg/kg). Administration of the anesthetics was repeated alternately every 30 min as required to maintain the animal in deep, stage IV anesthesia, as monitored by blood pressure and heart rate using a veterinary blood pressure monitor with a 5-cm pediatric cuff (model 9301V1; CAS Medical Systems). The deep anesthesia was needed to eliminate any eye reflexes during the acquisition of the images, which require the eye to be still for several seconds.

Intraocular pressure. IOP was determined before each hyperspectral imaging session using a tonometer (Tonopen XL; Medtronic). Before IOP determination, a topical anesthetic was instilled (proparacaine hydrochloride ophthalmic solution, 0.5%; Alcon) to reduce animal discomfort.

Oxygen saturation. To measure oxygen saturation of the ONH and paired retinal vessels, we placed a contact lens on the cornea to prevent drying and obtained reflectance hyperspectral imaging measurements in both eyes of each monkey.

Before dosing, baseline oxygen saturation for each region of interest was obtained. Each eye was then injected with 0.1 mL of NCX 434 or TA in the vitreous cavity at the specified dose (5.8 and 4.0 mg/eye for NCX 434 and TA, respectively) suspended in saline with 3% polyvinyl alcohol certified “sterile” and “endotoxin-free.” Oxygen saturation was determined at 7, 14, 21, 31, and 41 days postdosing in various ONH areas according to previously published protocols.¹³

Statistical methods

Least squares mean values for the site by pressure interaction means were tested for statistically significant differences using protected *t*-tests and an alpha level correction method based on a simulation method.

Results

In vitro characterization

³H-Dexamethasone displacement on hGR. This set of experiments was designed to monitor the steroid-like activity of NCX 434. The binding conditions were set up to obtain at least 80% of specific binding. NCX 434, with a displacement potency of 33.8 ± 3.0 nM, was slightly less active than TA ($IC_{50} = 5.9 \pm 1.8$ nM) (Fig. 2A).

Vasorelaxation in methoxamine-precontracted rabbit aortic rings. The ability of NCX 434 to induce *in vitro* vasorelaxation, indicative of NO release, was assessed on methoxamine-precontracted rabbit aortic rings using previously described method.²⁷ NCX 434 evoked concentration-dependent relaxation with an EC_{50} of 0.5 ± 0.03 μ M, achieving 98.9% relaxation at the highest concentration tested of 100 μ M. TA did not induce vasorelaxation at any concentration tested (Fig. 2B). The effects of NCX 434 were NO-dependent as the application of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μ M), an inhibitor of soluble

guanylyl cyclase, completely abolished the responses (data not shown).

Studies in the cynomolgus monkey

Oxygen saturation in ONH and overlying arteries and veins. Before compound testing, basal oxygen saturation percentage was obtained for the different ocular regions of interest. More specifically, the extent of oxygen saturation did not differ significantly in eyes later treated with 5.8 mg/eye of NCX 434 or 4.0 mg/eye of TA (percent oxygen saturation ranging between 61.9 ± 1.3 and 73.0 ± 1.4 or 64.7 ± 5.0 and 73 ± 3.3 for eyes later included, respectively, in the NCX 434 or TA group).

Similarly, within the large vein and conductance arteries, the extent of oxygen saturation did not significantly differ in eyes later treated with NCX 434 or TA (percent oxygen saturation, 80.9 ± 1.6 and 46.8 ± 1.4 or 81.1 ± 1.5 and 45.9 ± 3.5 for artery and vein compartments in eyes later injected with NCX 434 or TA).

The oxygen saturation percentage was then determined in the same regions at various time points (7, 14, 21, and 31

FIG. 2. (A) Binding properties on human glucocorticoid receptors (NCX 434, open triangle and TA, open square) and (B) nitric oxide-mediated vasorelaxation (Vehicle, open circle, NCX 434, open triangle and TA, open square) were tested, respectively, in recombinant human glucocorticoid receptors expressed in Sf9 insect cells and in isolated methoxamine-precontracted aortic rings. Each value represent the mean $\pm$ SEM of 3 individual experiments. TA, triamcinolone acetate.

FIG. 3. Oxygen saturation in optic nerve head following NCX 434 or TA IVT in (A-F) non-human primate. Oxygen saturation for the 6 regions following 5.8 mg/eye NCX 434 (open triangle) or 4.0 mg/eye TA (open square) IVT injection in cynomolgus monkey eyes. Values for each region represent the mean $\pm$ SE of 5 eyes each recorded 3 times. IVT, intravitreal.

FIG. 4. Oxygen saturation in overlying veins and arteries. Oxygen saturation within large ocular veins (closed triangle and closed square for NCX 434 and TA, respectively) or arteries (open triangle and open square for NCX 434 and TA, respectively) following NCX 434 (5.8 mg/eye) or TA (4.0 mg/eye) IVT injection in cynomolgus monkey eyes. Values for each region represent the mean $\pm$ SE of 5 eyes each recorded 3 times. * $p < 0.05$ vs. basal.

days) post-intravitreal (IVT) injection of the 2 compounds. As shown in Fig. 3, the IVT injection of 5.8 mg/eye of NCX 434 ($n=5$) caused significant increase in oxygen saturation percentage regardless of the specific region analyzed (Fig. 3A–F). The increase that was already registered at 7 days postinjection remained significantly above the basal value over the following weeks, and declined to baseline levels at later time points (Fig. 3A–F). These effects were not observed in any of the regions analyzed in eyes treated with an equimolar dose (4.0 mg/eye, $n=5$) of TA (Fig. 3A–F).

Conversely, oxygen saturation percentage decreased in the vein compartment and remained unaltered in the arteries of the NCX 434-treated but not the TA-treated group (Fig. 4). The effects were more evident at 7 and 14 days post-treatment and disappeared over the following weeks.

Intraocular pressure. Before injection of compound, IOP was monitored in eyes later treated with NCX 434 or TA and did not differ significantly between groups (10.3 ± 0.6 or

10.4 ± 0.7 mmHg for eyes later treated with NCX 434 or TA, respectively).

The IVT injection of NCX 434 did not affect IOP at all time points analyzed, whereas at 31 days following TA injection, though not significantly, the IOP tended to increase ($\Delta_{IOP} = +3.31 \pm 0.51$ mmHg, 30.8%, over baseline, NS) (Table 1).

Discussion

Glucocorticoids are among the most frequently used compounds for the treatment of certain ocular diseases²⁸ although for some pathologies such as DME, their use is not yet approved by the Food and Drug Administration. In DME, glucocorticoids readily reduce the inflammatory state and the edema but are not as efficient in reducing the retinal hypoxia and cell damage that often characterize this disease. Moreover, their administration has been shown to enhance the incidence of hypertensive glaucoma and cataract lesions in DME patients.⁹

NCX 434 is a new molecular entity designed to target DME as well as other ocular disorders associated with micro- and macrovascular abnormalities. The compound combines TA with an NO-donating residue readily released *in vivo* through the action of cellular esterases. This approach would assure glucocorticoid-mediated anti-inflammatory activity and NO-mediated vascular effects in target eye structures while keeping the IOP under control.

NCX 434 resulted in a sustained and long-lasting increase in oxygen saturation within the ONH in cynomolgus monkey eyes when the compound was administered intravitreally. Conversely, the administration of NCX 434 lowered oxygen saturation in veins, which is consistent with an increase in oxygen consumption within the surrounding tissues. This assumption is further strengthened by considering that the percentage of oxygen saturation in the branch retinal artery was not affected by the treatment. An interpretation of the above findings is that upon treatment with NCX 434, the saturation of the arteries is “soaked up” by the surrounding tissue; as a consequence, the saturation of the tissue rises and only that remaining proceeds through the veins, resulting in a lower saturation level in this specific compartment.

These effects might not be due to the glucocorticoid signaling pathway as the reference TA administered at equimolar doses did not result in any significant change in ONH or in the overlying vessels. In our study, the extent of oxygen saturation in vessels under basal conditions was 9%–10% lower than that found by other investigators.²⁹ This was

TABLE 1. INTRAOCULAR PRESSURE FOLLOWING NCX 434 OR TRIAMCINOLONE ACETONIDE INTRAVITREAL INJECTION IN CYNOMOLGUS MONKEYS

NCX 434 (5.8 mg/eye)			Triamcinolone acetonide (4.0 mg/eye)	
Day	IOP (mmHg)	Δ_{IOP} (vs. baseline) (mmHg)	IOP (mmHg)	Δ_{IOP} (vs. baseline) (mmHg)
Basal	10.3 ± 0.3	—	10.4 ± 0.3	—
14	10.6 ± 0.4	0.3 ± 1.0	10.7 ± 0.5	0.3 ± 0.5
21	11.9 ± 0.5	1.4 ± 1.3	11.6 ± 0.8	1.2 ± 1.8
31	11.5 ± 0.3	1.3 ± 0.6	13.5 ± 0.2	3.1 ± 0.5

Intraocular pressure (IOP) was determined 15 min after anesthesia was induced using a tonometer (Tonopen XL; Medtronic). Proparacaine hydrochloride ophthalmic solution 0.5% was used as local anesthesia to further reduce animal discomfort. Values represent mean $\pm$ SEM of 5 eyes each measured 3 times at 5-min intervals and averaged.

likely to be due to the deep anesthesia, which was needed to maintain stability of the eye during hyperspectral imaging data collection. As with the vessels, the tissue values we report may also be lower than those that would otherwise be found in animals that are awake.

The cellular and molecular mechanisms involved are far from being elucidated. NCX 434 resulted in vasodilatation in isolated rabbit aortic rings, suggesting that this compound might also induce vasodilation within the ocular compartments and, as a consequence of this, increase the oxygen delivered to ONH. This is consistent with previous work²⁴ demonstrating that NCX 1021, an NO-donating dexamethasone, by virtue of its NO-donating properties, lowers the ophthalmic artery resistance index in rabbits.

As an additional end-point, we also monitored IOP. Compared to previous studies, IOP in our animals was relatively low. This is explained considering that the measurements were taken while the animals were under deep anesthesia. In this condition we did not record changes over time following the administration of NCX 434 or TA. However, though not significantly, a considerable IOP increase (30%) was noted in the TA-treated group at the later time points, which is consistent with the effects of IVT triamcinolone observed in humans.^{9,28} NO lowers IOP in experimental animals^{14,23} and humans^{29,30} by enhancing the outflow capacity. Thus, it is conceivable that the absence of IOP-raising effects for NCX 434 is consequent to the activation of the NO/cGMP signaling pathway in the trabecular meshwork and Schlemm's canal. Additional studies combining cGMP and IOP measurements in awake animals are needed to better address this specific aspect.

In conclusion, these findings suggest that NCX 434, by virtue of its NO-donating properties, enhances ocular tissue oxygenation without affecting IOP. These features make it an important compound to be further investigated for the treatment of DME and other ocular disorders with impaired ocular perfusion.

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Author Disclosure Statement

V. Chirolì, D. Ronchetti, D. Miglietta, E. Ongini, and F. Impagnatiello were employed at Nicox Research Institute at the time this work was performed.

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