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NCX 470 Restores Ocular Hemodynamics and Retinal Cell Physiology After ET-1-Induced Ischemia/Reperfusion Injury of Optic Nerve and Retina in Rabbits

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Abstract

Purpose: Determine whether NCX 470, a nitric oxide (NO)-donating bimatoprost with clinically demonstrated intraocular pressure (IOP)-lowering effects, improves ocular hemodynamics and retinal physiology.
Methods: Endothelin-1 (ET-1)-induced ischemia/reperfusion model in New Zealand white rabbits was used. ET-1 was injected next to the optic nerve twice/week (Monday and Thursday) for 6 weeks. Starting on week 3, animals received NCX 470 (0.1% bid, 6 days/week Monday–Saturday) or vehicle until the end of ET-1 treatment. IOP, ophthalmic artery resistive index (OA-RI) and retina physiology (electroretinogram, ERG) were determined before dosing and at different times post-dosing. All measurements were taken on Mondays before the AM daily dosing (36 h treatment-free). Finally, oxidative stress markers were determined in dissected retina and iris/ciliary body of treated eyes.
Results: Injection of ET-1 progressively increased IOP (20.7 ± 0.6, 24.9 ± 1.2, and 27.0 ± 0.6 mmHg at baseline, week 2 and 6, respectively) and OA-RI (0.30 ± 0.02, 0.39 ± 0.02, and 0.42 ± 0.03 at baseline, week 2 and 6, respectively) and reduced rods and/or cones response as indicated by changes in ERG amplitudes under different stimulating conditions. NCX 470 re-established baseline IOP (21.8 ± 1.0 mmHg), OA-RI (0.33 ± 0.02), and ERG amplitude by week 6 (mostly rod response, $^{0.01}\text{Dark\_A}_{\text{Veh\_6week}} = 32.2 \pm 3.0\,\mu\text{V}$ and $^{0.01}\text{Dark\_A}_{\text{NCX470\_6week}}$ $44.3 \pm 4.5\,\mu\text{V}$; mostly cone response, $^{3.0}\text{Dark\_A}_{\text{Veh\_6week}} = 87.6 \pm 10.1\,\mu\text{V}$ and $^{3.0}\text{Dark\_A}_{\text{NCX470\_6week}} = 122.8 \pm 11.4\,\mu\text{V}$; combined rod/cone response, $^{3.0}\text{Light\_A}_{\text{Veh\_6week}} = 49.8 \pm 6.5\,\mu\text{V}$ and $^{3.0}\text{Light\_A}_{\text{NCX470\_6week}} = 64.2 \pm 6.8\,\mu\text{V}$). NCX 470 also reversed ET-1-induced changes in glutathione and manganese superoxide dismutase (oxidative stress markers) in retina and iris/ciliary body.
Conclusions: Repeated ocular topical dosing with NCX 470 reverses ET-1-induced changes in IOP, OA-RI, and ERG suggesting improved ocular hemodynamics and retinal physiology likely independently from its demonstrated IOP-lowering effect.

Keywords: nitric oxide, prostaglandin, ischemia/reperfusion, ocular hemodynamics, photoreceptor function

Introduction

NCX 470 is a nitric oxide (NO)-donating bimatoprost derivative in clinical phase 3 for the lowering of intraocular pressure (IOP) in patients with ocular hypertension or open-angle glaucoma. Upon administration NCX 470 is rapidly cleaved into 2 active metabolites, bimatoprost and the NO-donating moiety, 6-(nitrooxy)hexanoic acid that ultimately releases NO.[1]

Although there is evidence for the existence of an as-yet-unidentified specific prostamide receptor,[2] our current understanding indicates that bimatoprost, despite its prostamide

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nature, mainly acts *via* the prostaglandin FP receptors.[3] Separately, NO stimulates soluble guanylyl cyclase activity with consequent cyclic guanosine monophosphate (cGMP) formation in the eye.[4–6] As a result, NCX 470 lowers IOP in animals and patients by promoting both (1) bimatoprost–mediated aqueous humor (AH) drainage through the interstitial spaces of the ciliary muscle, supra-choroidal space of the eye, and finally the capillaries and lymphatic vessels (this pathway is referred to as the unconventional or uveoscleral outflow pathway)[7,8]; and (2) NO-mediated increase in outflow via cGMP-dependent relaxation of the trabecular meshwork and Schlemm's canal (this pathway is referred to as the conventional, pressure-dependent outflow pathway).[4,5]

Currently lowering IOP is the only approved pharmacotherapy to treat glaucoma patients,[9] however, a subset of patients exhibits optic neuropathy associated with visual field loss despite normal IOP[10]; and conversely, not all patients with high IOP develop glaucoma. Furthermore, reduction of IOP, while lowering the risk of glaucoma progression, does not entirely stop retinal and optic nerve degeneration.[11]

Growing evidence suggests that vascular factors may play a role in the pathogenesis of this disease.[12] Specifically, retinal ganglion cell loss is often attributed to insufficient blood supply to the retina and optic nerve head where vascular tone is strictly regulated by the balanced release of vasoactive factors. These vasoactive factors are namely endothelins [endothelin-1 (ET-1), ET-2, and ET-3], known to have potent vasoconstricting activity and inhibitory effects on endothelial permeability in the eye; and NO, a vasodilator typically released in the eye by the vascular endothelium that forms a single layer of cells at the interface between the vessel wall and the blood stream, or released by the endothelial cells lining all trabecular meshwork channels and Schlemm's canal that forms a single layer of cells at the interface between the vessel wall and the blood.[13]

Among endothelins, ET-1, a peptide of 21 amino acids, acts *via* two G-protein-coupled receptors: the ET_A receptors, expressed in multiple ocular tissues including ciliary body, trabecular meshwork, retina, and lamina cribrosa,[14] are known to control microvascular contractility and to regulate trabecular meshwork contractility and outflow facility[15]; and, ET_B receptors typically located on endothelial cells and on smooth muscles where they appear to counterbalance the effects of ET_A by inducing vasorelaxation *via* the release of NO.[16]

There is evidence of a clear relationship between ET-1 system dysfunction in the eye and glaucoma pathophysiology. Studies have shown that ET-1 concentrations are significantly increased in the AH and plasma of glaucomatous patients,[17,18] and in animal models of glaucoma.[19] Likewise, ET-1 receptor mRNA expression is increased in retina after IOP elevation in a rat model of glaucoma.[20] The use of ET-1 is well established in animal experiments to mimic glaucoma retinopathy and study the effects of ischemic injury on metabolically active tissues like the retina and optic nerve. In addition, in this same model the restoration of blood flow between consecutive injections of ET-1 elicits a cascade of events that ultimately leads to additional cell injury known as reperfusion injury.[21,22] We have previously used the ET-1-induced ischemia/reperfusion model in rabbits and we showed that all ET-1-induced detrimental effects on ocular hemodynamics and retinal dysfunction were partially reversed by NO.[22]

Here we used the same rabbit model to address the effects of NCX 470 on ET-1-induced biochemical oxidative stress markers and functional changes including IOP, vascular reactivity, and retinal response to flash light stimuli in different experimental conditions to differentiate rod from cone responses. Findings would indicate that NCX 470, independently from its demonstrated IOP-lowering ability, also exerts vascular effects and improves retinal function in this experimental animal model.

Methods

Animals

A total of 16 adult male New Zealand white (NZW) rabbits weighting 1.5–2.0 kg were used. The experimental procedures conformed with those of the Association for Research in Vision and Ophthalmology Resolution on the use of the animals and were conducted in accordance with the Italian regulation on protection of animals used for experimental and other scientific purpose (Italian Legislative Decree 26, March 13, 2014) and with the EU Regulations (Council Directive of the European Community 2010/63/EU). The study was also approved by the local animal care committee of the University of Florence (Italy) and followed Ministry of Health recommendations (authorization n. 110/2021-PR). Every effort was made to minimize animal suffering and to reduce the number of animals used. The animals were kept in individual cages, food and water were provided *ad libitum*. The animals were identified with a tattoo on the ear, numbered consecutively, and housed on a 12–12 h light/dark cycle in a temperature controlled room (22°C–23°C).

Experimental animal model and test item dosing

The ET-1-induced ischemia/reperfusion model was used. Ischemia/reperfusion injury was induced in each animal by subtenon injection (twice/week for 6 weeks) of 200 μL of 250 nM ET-1 (Fluka, Israel) dissolved in water using a lacrimal cannula under anesthesia produced by ketamine and xylazine injected intramuscularly.[22] After ET-1 injection, a drop of tobramycin (0.3% ophthalmic solution) was instilled in each eye.

NCX 470 (0.1%, 30 μL/eye) or vehicle (30 μL/eye) were administered as eye drops, bid (at ~10:00 AM and 4:00 PM) for 4 consecutive weeks starting on week 3 concomitantly to ET-1 until week 6 by a treatment-masked investigator.

Biochemical determinations

Retina and iris/ciliary body were collected from all animals at the end of the study and from naïve animals for comparative reasons. Animals were euthanized with an overdose of anesthetic (pentothal sodium 0.15 g/kg, iv bolus) and tissues dissected and frozen until further processing.

Glutathione level. Retina and iris/ciliary body were homogenized in 2 mL of 10 mM phosphate buffer, pH 7.4 and then centrifuged at 10,000*g* for 30 min at 4°C, then a fixed volume of supernatant was diluted with phosphate buffer added with ethylene diamine tetraacetic acid (EDTA) to

2 mL. The resulting solution was then processed to determine the levels of reduced glutathione (GSH). Briefly, 200 μL of 40 mM N-ethylmalemide (Sigma-Aldrich Chemie, Steinheim, Germany) were added to 0.5 mL of sample, incubated for 30 min, and diluted with 0.1 mL of NaOH 0.1 N and 1.8 mL phosphate-ethylenediamine tetraacetic acid (EDTA) buffer; at the end 0.1 mL σ-phthaldialdehyde (Analyticals, Carlo Erba, Milan, Italy, 1 mg/mL in methanol, Uvasol) was added. After 20 min incubation at room temperature the fluorescence of the samples was read at 420 nm emission with 350 nm excitation. The content of GSH was determined against a standard curve and normalized on protein content.

Manganese superoxide dismutase activity. Retina and iris/ciliary body were homogenized and centrifuged as described above. Manganese superoxide dismutase (MnSOD) activity was determined in a fixed volume of supernatant according to a previously described method.[22]

Briefly, a competitive inhibition assay that used xanthine-xanthine oxidase-generated superoxide to reduce nitro blue tetrazolium (NBT; Sigma) to blue tetrazolium was used. The reaction was performed in a final volume of 2 mL, containing 100 μL of the sample, 1.7 mL of 50 mM sodium carbonate-EDTA (0.1 mM) buffer, pH 10.1, 100 μL xanthine (Sigma) 0.1 mM, and 100 μL NBT 25 μM. In a spectrophotometer (Perkin Elmer Lambda 5; Perkin Elmer Life and Analytical Sciences, Milan, Italy), at a wavelength of 560 nm, 50 μL xanthine oxidase (Sigma) was added to each sample and the reduction of NBT monitored for 30 s. The standard curve was performed in a sample free system containing increasing concentration of MnSOD. The amount of protein required to inhibit the rate of NBT reduction by 50% was defined as 1unit (U) of enzyme activity. The values are expressed as mU and normalized on protein content.

Functional assessments

Out of the 16 animals used in this study, 10 animals (left and right eyes were randomly assigned to vehicle or NCX 470) were treated and tested for IOP and ophthalmic artery resistive index (OA-RI), whereas the remaining ($n=6$) animals although receiving similar treatment were utilized to address photoreceptor function (ERG).

IOP determinations

IOP was measured at the indicated time points using a pneumatonometer (Model 30 Classic; Reichert, Depew, NY) on Mondays (36 h free of treatment) before AM dosing with test items. One drop of oxybuprocaine hydrochloride (4 mg/mL) was instilled immediately before each set of pressure measurements. Values reported for each time point were the mean of 2 consecutive measurements taken 1 min apart. IOP changes from baseline were calculated as follows: IOP_{Tx}–IOP_{T0} where IOP_{Tx} and IOP_{T0} are, respectively, the IOP at the time of interest and at baseline.

Ophthalmic artery resistive index

Measurements were taken using an Echo Color Doppler (Philips Ultrasound HD7XE; Philips, Milan Italy) with the S12–4 ultrasound transducer and a frequency of 6.0 MHz, before ET-1 treatment (baseline, time 0), and weekly thereafter on Mondays until the end of the study. Pourcelot resistive index for ophthalmic artery (OA-RI) was calculated using the following formula: (OA-PSV–OA-EDV)/OA-PSV where OA-PSV and OA-EDV refer to ophthalmic artery peak systolic velocity and ophthalmic artery end diastolic velocity, respectively.[23] Representative images in Supplementary Fig. S1.

Electroretinogram

Topical anesthesia was achieved using 1 drop of oxybuprocaine hydrochloride (4 mg/mL). The eyes were then dilated by topical application of tropicamide 1% and, when needed, adapted to darkness for at least 2 h before standard electroretinograms (ERGs) recording of both eyes using contact lens corneal electrodes so to have sufficiently stable and amplified recordings. The ERG signals were recorded using Retimax (CSO, Florence, Italy) and according to the current International Society for Clinical Electrophysiology (ISCEV) indications.[24] Specifically the dark-adapted 0.01 ERG (mostly rod response), dark-adapted 3.0 ERG (combined rod/cone response), and light-adapted 3.0 (mostly cone response) were recorded. Flashes' intensity varied:

(a) from 0.01 photopic cd.s.m^{-2} to 0.025 scotopic cd.s.m^{-2} with a minimum interval between flashes of 2 s for the dark-adapted 0.01 ERG;
(b) from 3.0 photopic cd.s.m^{-2} to 7.5 scotopic cd.s.m^{-2} with a minimum interval between flashes of 10 s for the dark-adapted 3.0 ERG;
(c) from 3.0 photopic cd.s.m^{-2} to 7.5 scotopic cd.s.m^{-2} with a minimum interval between flashes of 0.5 s and a light adaptation strength of 30 cd.s.m^{-2} for the light-adapted 3.0 ERG. In each case ERG recording lasted 250 ms.

Measurements were taken before ET-1 first dose (baseline, time 0), then at the end of week 2 (before vehicle- or NCX 470-first day-first dose) and at the end of week 6 (36 h after vehicle or NCX 470-last day-last dose). Representative traces in Supplementary Fig. S2.

Results

IOP changes following repeated NCX 470 dosing after ET-1-induced ischemia/reperfusion injury in NZW rabbits

Average baseline IOP before ET-1 dosing was 20.7 ± 0.6 mmHg and 21.3 ± 0.5 mmHg respectively in animals later randomized for vehicle or NCX 470 treatments (Fig. 1A, B). Twice weekly dosing with ET-1 for 2 weeks raised average IOP to reach 24.9 ± 1.2 and 26.4 ± 1.5 mmHg in animals intended for vehicle and NCX 470 groups, respectively (Fig. 1A, B).

NCX 470 daily dosing (0.1%, 30 μL/eye bid) progressively counteracted ET-1-induced changes as documented by the decrease in IOP observed in treatment-free conditions (36 h after last treatment) to reach 21.8 ± 1.0 mmHg at week 6. IOPs in vehicle-treated animals remained stable over the same period (at week 6, 27.0 ± 0.6 mmHg) (Fig. 1A, B). The administration of NCX 470 did not result in evident

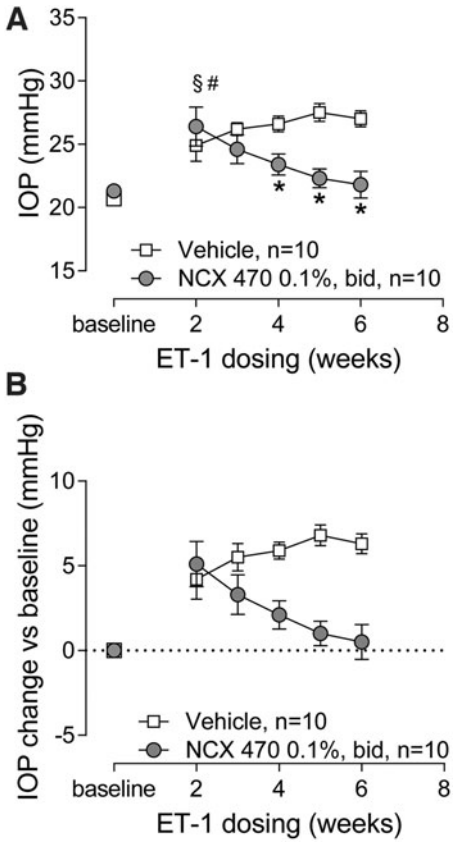

FIG. 1. IOP changes after NCX 470 repeated dosing after ET-1-induced ischemia/reperfusion injury in NZW rabbits. **(A)** IOPs, and **(B)** IOP changes from baseline, after instillation of NCX 470 (0.1%, bid, $n=10$) or vehicle ($n=10$) for 4 consecutive weeks starting 2 weeks after ET-1 first dosing. IOPs were measured using a pneumatonometer. IOP changes versus baseline were calculated as follows: $(IOP_{Tx}-IOP_{T0})$ where IOP_{Tx} and IOP_{T0} are, respectively, the IOP at the time of interest and at baseline. Data are reported as mean ± SEM. $^{*}P<0.05$ versus vehicle at the same time point. $^{\S,\#}P<0.05$ versus respective baselines, *t*-test multiple comparisons. ET-1, endothelin-1; IOP, intraocular pressure; NZW, New Zealand white; SEM, standard error of the mean.

ocular side effects during the entire experimental period as determined by daily visual inspection before and after NCX 470 or vehicle dosing (data not shown).

OA-RI changes after NCX 470 repeated dosing following ET-1-induced ischemia/reperfusion injury in NZW rabbits

PSV and EDV were measured over time. Data were then computed and used to calculate the respective OA-RI. The OA-RI before ET-1 dosing was 0.30 ± 0.02 and 0.30 ± 0.02 respectively in animals later randomized for vehicle or NCX 470 treatments (Fig. 2A). Twice weekly dosing with ET-1 for 2 weeks raised OA-RI equally in both groups (0.39 ± 0.02 and 0.36 ± 0.03 for vehicle and NCX 470, respectively) (Fig. 2A).

In animals treated with vehicle, the OA-RI further increased over the following 4 weeks (0.42 ± 0.02 and 0.42 ± 0.03 on week 5 and 6, respectively). In contrast, OA-RI taken under treatment-free conditions in NCX 470 (0.1%)-treated animals decreased significantly (0.32 ± 0.03 and 0.33 ± 0.02 on week 5 and 6, respectively) (Fig. 2A).

Notably, only PSV tended to decrease over time in NCX 470-treated animals, although not significantly, with no major changes in EDV (Fig. 2B, C).

ERG changes following NCX 470 repeated dosing after ET-1-induced ischemia/reperfusion injury in NZW rabbits

In the ERG, the a-wave is a negative, maximal response that is believed to reflect the membrane potential in photoreceptors and thus their integrity. More specifically, depending on the light adaptation and the intensity/frequency of the flash light stimulation used, a-waves are more representative of mostly rod (dark adapted, scotopic—low intensity/frequency light flash stimulation), cone (light-adapted, photopic—high intensity/frequency light flash stimulation), or combined rod and cone (dark adapted, scotopic—high intensity/frequency light flash stimulation) responses. Conversely, the b-wave typically registered in the ERG is a positive, maximal combined response that is thought to originate from the Bipolar and Müller cells that are post-synaptic to photoreceptors.

In our study, ERGs were performed under all 3 stimulating conditions for both NCX 470- and vehicle-treated eyes before ET-1 first dosing (baseline, time 0), then at the end of week 2 (before vehicle- or NCX 470-first day-first dose) and at the end of week 6 (36 h after vehicle- or NCX 470-last day-last dose) in eyes that received twice weekly dosing of ET-1.

Dark adapted scotopic ERG 0.01—rod response (Amplitude, $^{0.01}$Dark_A; Peak Latency, $^{0.01}$Dark_P). The mean ERG amplitude (*b-wave* amplitude minus *a-wave* amplitude) and the respective latencies (*b-wave* latency minus *a-wave* latency) after dark-adapted scotopic ERG 0.01 stimulation are shown in Table 1 and Fig. 3A.

Baseline scotopic ERG response was similar in animals later randomized for vehicle or NCX 470 treatment both as amplitude ($^{0.01}Dark\_A_{Veh\_BASELINE}=49.3\pm4.7\,\mu V$ and $^{0.01}Dark\_A_{NCX470\_BASELINE}=42.3\pm6.9\,\mu V$) and peak latency ($^{0.01}Dark\_P_{Veh\_BASELINE}=31.0\pm1.9$ msec and $^{0.01}Dark\_P_{NCX470\_BASELINE}=31.5\pm2.2$ msec). ERG amplitude declined substantially after 6-weeks of twice weekly ET-1 treatment ($^{0.01}Dark\_A_{Veh\_6week}=32.2\pm3.0\,\mu V$, $P<0.05$ vs. basal) without major changes in peak time ($^{0.01}Dark\_P_{Veh\_6week}=23.6\pm2.3$ msec). Interestingly, dosing the animals for 4 consecutive weeks with NCX 470 (0.1%, bid) abolished ET-1-induced ERG amplitude decline ($^{0.01}Dark\_A_{NCX470\_6week}=44.3\pm4.5\,\mu V$) without major changes in peak time ($^{0.01}Dark\_P_{NCX470\_6week}=33.9\pm7.0$ msec) (Table 1).

Dark adapted, scotopic ERG 3.0—rod/cone response (Amplitude, $^{3.0}$Dark_A; Peak Latency, $^{3.0}$Dark_P). Dark adapted, scotopic ERG 3.0 amplitude (*b-wave* minus *a-wave*) representative of the combined rod/cone activity is shown in Table 1 and Fig. 3B. Combined rod and cone response did not differ in eyes later randomized for vehicle or NCX 470. Specifically, scotopic response amplitudes recorded at high frequency/intensity stimulation at baseline

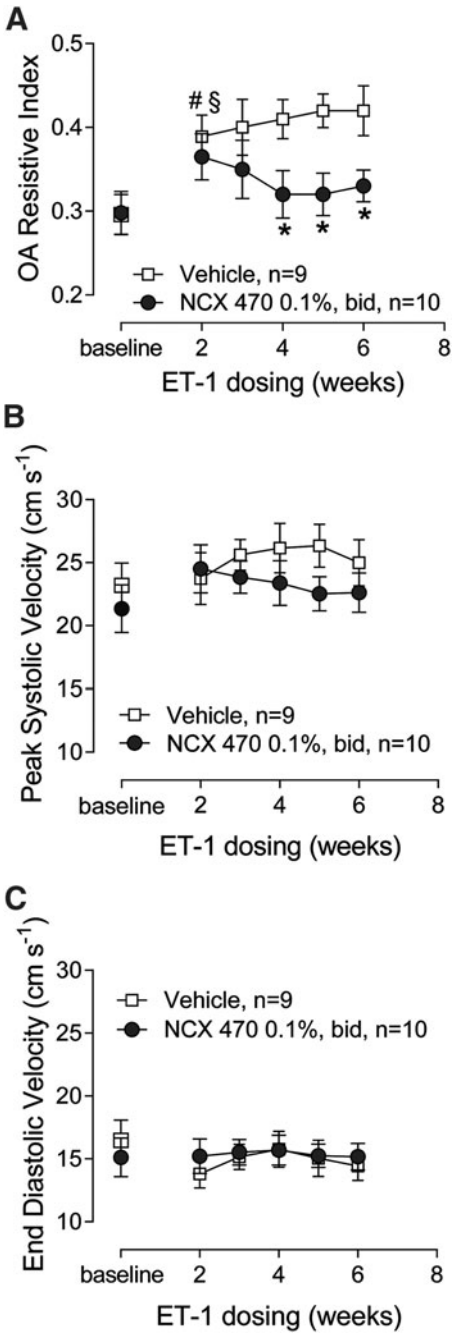

FIG. 2. OA-RI changes after NCX 470 repeated dosing following ET-1-induced ischemia/reperfusion injury in NZW rabbits. **(A)** OA-RI; **(B)** PSV and, **(C)** EDV after instillation of NCX 470 (0.1%, bid, $n=10$) for 4 consecutive weeks starting 2 weeks after ET-1 first dosing and the respective vehicle ($n=9$). OA-RI was calculated with the following formula: (PSV–EDV)/PSV. *$P<0.05$ versus vehicle at the same time point. §,# $P<0.05$ versus respective baselines, *t*-test multiple comparisons. EDV, ophthalmic artery end diastolic velocity; PSV, ophthalmic artery peak systolic velocity; OA-RI, ophthalmic artery resistive index.

was $^{3.0}\text{Dark\_A}_{\text{Veh\_BASELINE}}=109.8\pm12.9\,\mu\text{V}$ in eyes later randomized for vehicle and $^{3.0}\text{Dark\_A}_{\text{NCX470\_BASELINE}}=118.0\pm10.3\,\mu\text{V}$ for eyes later treated with NCX 470. Responses declined substantially in ET-1-treated eyes at week 6 ($^{3.0}\text{Dark\_A}_{\text{Veh\_6week}}=87.6\pm10.1\,\mu\text{V}$, $P<0.01$ vs. baseline) in eyes receiving vehicle. NCX 470 treatment counteracted the detrimental effects of ET-1 ($^{3.0}\text{Dark\_A}_{\text{NCX470\_6week}}=122.8\pm11.4\,\mu\text{V}$, $P<0.01$ vs. vehicle). Peak time was not affected by ET-1 treatment and remained substantially unchanged after vehicle or NCX 470 dosing (Table 1).

Light-adapted, photopic ERG 3.0—cone response (Amplitude, $^{3.0}$Light_A; Peak Time, $^{3.0}$Dark_P). The mean ERG amplitude (*b-wave* minus *a-wave*) after light-adapted, photopic ERG 3.0 stimulation is reported in Table 1 and Fig. 3C. As in the previous measures described above, baseline amplitude was similar in eyes later randomized for vehicle or NCX 470 ($^{3.0}\text{Light_A}_{\text{Veh_BASELINE}}=70.4\pm8.8\,\mu\text{V}$ and $^{3.0}\text{Light\_A}_{\text{Veh\_NCX470}}=67.6\pm6.4\,\mu\text{V}$, respectively). Eyes receiving vehicle concomitantly with ET-1 twice weekly had, at week 6, although not significantly, lower response ($^{3.0}\text{Light\_A}_{\text{Veh\_6week}}=49.8\pm6.5\,\mu\text{V}$). Repeated dosing with NCX 470 (4 weeks, 0.1% bid) tended to counteract ET-1 effects ($^{3.0}\text{Light\_A}_{\text{NCX470\_6week}}=64.2\pm6.8\,\mu\text{V}$). No major changes were observed in mean peak time irrespective of treatment (Table 1).

Oxidative stress markers following NCX 470 repeated dosing after ET-1-induced ischemia/reperfusion injury in NZW rabbits

Levels of GSH were 1,295.6 ± 25.2 and 2,160.2 ± 710.0 pmoles/μg of proteins in retina and iris/ciliary body, respectively (Fig. 4A). MnSOD activity was 31.1 ± 1.6 and 22.3 ± 3.8 mU/μg of proteins in retina and iris/ciliary body, respectively (Fig. 4B).

Eyes receiving ET-1 for 2 weeks followed by additional 4 weeks of treatment with vehicle had lower GSH levels in retina and iris/ciliary body (371.5 ± 43.7 and 1,213.2 ± 79.3 pmoles/μg of proteins in retina and iris/ciliary body, respectively, $P<0.05$) compared to naïve eyes. Likewise, MnSOD activity was also lower in retina and iris/ciliary body of vehicle eyes (18.9 ± 2.3 and 17.5 ± 1.2 mU/μg of protein in retina and iris/ciliary body, respectively, $P<0.05$) compared to naïve eyes (Fig. 4B). Interestingly, eyes treated with NCX 470 had GSH levels similar to naïve eyes both in retina (1,314.6 ± 253.0 pmoles/μg of proteins, $P<0.05$ vs. vehicle) and iris/ciliary body (3,158.1 ± 256.3 pmoles/μg of proteins, $P<0.05$ vs. vehicle) (Fig. 4A); in addition, MnSOD activity was re-established after NCX 470 treatment (30.1 ± 3.0 and 22.1 ± 1.4 mU/μg of protein, respectively in retina and iris/ciliary body, $P<0.05$ vs. vehicle) in both tissues (Fig. 4B).

Discussion

NCX 470 is a new molecular entity[25] comprising the prostamide bimatoprost esterified at the hydroxyl group in position 15 with the NO-donating moiety 6-(nitrooxy)hexanoic acid.[1] The compound holds promise to become the best-in-class IOP-lowering agent based on a combination of 2 converging modes of action both targeting IOP reduction, (1) prostaglandin F2α-mediated uveoscleral outflow activation and (2) NO-dependent trabecular meshwork and Schlemm's canal relaxation leading to increased conventional outflow capacity.[1]

ET-1-induced ischemia/reperfusion injury of the optic nerve and retina in rabbits shares many characteristics

Table 1. Electroretinogram Responses After NCX 470 Repeated Dosing Following Endothelin-1-Induced Ischemia/Reperfusion Injury in New Zealand White Rabbits

		Dark-adapted 0.01 rod responses		*Dark-adapted 3.0 rod/cone responses*		*Light-adapted 3.0 cone responses*	
		Amplitude $^{0.01}Dark\_A$ *(μV)*	*Latency* $^{0.01}Dark\_P$ *(msec)*	*Amplitude* $^{3.0}Dark\_A$ *(μV)*	*Latency* $^{3.0}Dark\_P$ *(msec)*	*Amplitude* $^{3.0}Light\_A$ *(μV)*	*Latency* $^{3.0}Light\_P$ *(msec)*
Vehicle	Basal	49.3 ± 4.7	31.0 ± 1.9	109.8 ± 12.9	21.8 ± 0.2	70.4 ± 8.8	15.3 ± 0.6
	2-weeks	38.0 ± 3.4	28.8 ± 1.5	98.6 ± 6.6	21.4 ± 0.5	59.9 ± 2.9	14.6 ± 0.4
	6-weeks	32.2 ± 3.0*	26.3 ± 2.3	87.6 ± 10.1	21.6 ± 0.4	49.8 ± 6.5	15.0 ± 0.4
NCX 470	Basal	42.3 ± 6.9	31.5 ± 2.2	118.0 ± 10.3	21.9 ± 0.4	67.6 ± 6.4	15.5 ± 0.7
(0.1%)	2-weeks	37.6 ± 6.2	27.4 ± 2.4	96.6 ± 9.4	20.2 ± 1.4	60.2 ± 4.3	15.0 ± 0.1
	6-weeks	44.3 ± 4.5#	33.9 ± 7.0	122.8 ± 11.4#	21.3 ± 0.3	64.2 ± 6.8	14.4 ± 0.7

Data are expressed as mean ± SEM. All animals received a 30 μL drop per eye of NCX 470 (0.1%, $n = 6$) or vehicle ($n = 6$) twice daily (10:00 AM and 4:00 PM, Monday to Saturday) for 4 consecutive weeks starting 2 weeks after ET-1 first dosing. Before measurements, animals were adapted to darkness for 2 h and scotopic assessments were obtained using flash light stimulation. Amplitude was calculated as the difference between b-wave and a-wave. Latencies were calculated from the peak a-wave to peak b-wave. *$P < 0.05$ versus respective baseline, #$P < 0.05$ versus vehicle, *t*-test multiple comparisons.

ET-1, endothelin-1; SEM, standard error of the mean.

also observed in glaucoma patients including IOP increase, ocular hemodynamic changes, and retinal cell dysfunction; thus it represents an ideal model to study potential NCX 470-mediated effects beyond its IOP-lowering ability.

Twice weekly retrobulbar injections of ET-1 (200 μL, 250 nM) resulted in progressive, stable increase in IOP and in important ocular hemodynamic changes evidenced by increased OA-RI (as detected by Echo Color Doppler imaging) accompanied by a progressive decline of photoreceptor function involving both rods and cones components. The ERG response recorded after flash light stimulations in scotopic or photopic conditions decreased over time, to the greatest extent 6 weeks after the initial dose.

Four weeks of bid dosing with NCX 470 progressively diminished ET-1-induced IOP elevation. In a previous non-human primate study, NCX 470-mediated IOP lowering after a single topical drop lasted up to 18–24 h[1] and the resulting NCX 470 exposure of ocular tissues including AH, cornea and iris/ciliary body was below the limit of detection at all time points; yet active bimatoprost acid, although low, was still detectable 18 h post dosing.[1] Interestingly, in that same study the downstream signaling marker of NO, cGMP, increased over time to reach maximum 24 h after NCX 470 treatment.[1]

In the present study the effects of NCX 470 on IOP were recorded after 4-weeks repeated bid dosing thus, although unlikely, residual prostaglandin F2α- and/or NO-like activities could have been still present at the time measurements were taken. Rabbits are relatively insensitive to prostaglandin F2α effects on uveoscleral outflow.[1,26] Furthermore, sustained exposure to bimatoprost *via* an intracameral implant have been shown to reduce episcleral venous pressure (EVP) and consequently decrease IOP,[27] a signaling pathway potentially also affected by NO, suggesting that similar mechanism involving EVP modulation could be responsible for the IOP effects seen in the present study.

The OA-RI is a ratio that relates PSV to EDV and represents the resistance to blood flow distal to the site of measurement. OA-RI and PSV and EDV are altered in glaucoma patients.[28,29] Repeated dosing with NCX 470 significantly reversed the changes in OA-RI induced by ET-1 over time and these effects could not be associated to a specific change in PSV or EDV; however, PSV appeared to increase after ET-1 treatment while it remained constant after concomitant dosing with NCX 470. Conversely, EDV decreased slightly after ET-1 and remained as such regardless of whether the animals were later co-administered with NCX 470 or vehicle. The minor changes in EDV and PSV might be due to the short (6-weeks) ET-1 protocol duration and/or ET-1 dose; a longer ET-1 treatment with larger doses might be necessary to see major changes in these two parameters and consequently explore to what extent they are modulated by NCX 470.

Prostaglandin F2α stimulation has been shown to either be ineffective[30] or to increase ocular blood flow.[31] Likewise, NO is a major regulator of ocular blood flow in healthy and glaucomatous eyes.[32] Thus both active metabolites, bimatoprost acid and NO, could have contributed to these effects. Consistent with that, Vyzulta® (latanoprostene bunod ophthalmic solution, 0.024%), a Food and Drug Administration (FDA)-approved drug for the lowering of IOP in patients with ocular hypertension or glaucoma, which similarly has an NO-donating moiety bound to a prostaglandin agonist as NCX 470, has been shown to enhance ocular perfusion pressure in glaucoma patients.[33]

NCX 470 also reversed ET-1-induced changes in retinal function. Specifically, eyes treated with NCX 470 had significantly less impairment in rod response (dark-adapted scotopic ERG 0.01) and in the overall rod/cone response (dark-adapted scotopic ERG 3.0) compared to vehicle. In addition, we found that cone response (light-adapted photopic ERG 3.0), although not significantly, was numerically different from vehicle. As for the hemodynamic changes, ERG differences were also observed 36 h after the last drug administration. Prostaglandins including tafluprost, latanoprost, and bimatoprost have been shown to be neuroprotective in various animal species.[34,35] Conversely, depending on the circulating concentrations, both neurodegeneration and neuroprotection has been reported for NO; for instance, while some

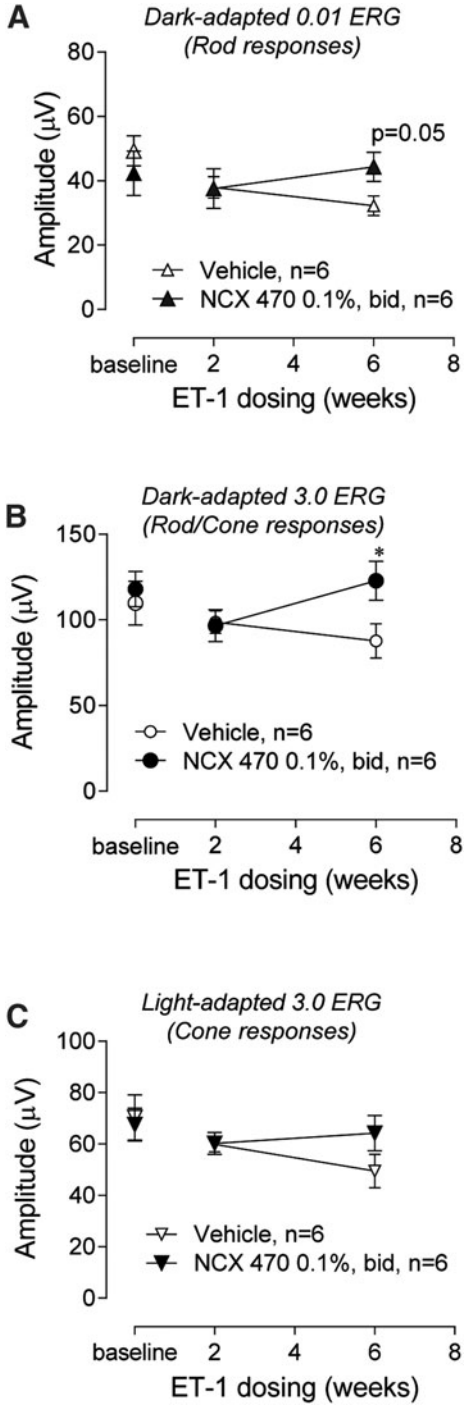

FIG. 3. ERG after NCX 470 repeated dosing following ET-1-induced ischemia/reperfusion injury in NZW rabbits. ERG responses after **(A)** dark-adapted 0.01 flash light stimuli; **(B)** dark-adapted 3.0 flash light stimuli; and **(C)** light-adapted 3.0 flash light stimuli after instillation of NCX 470 (0.1%, bid, $n=6$) or vehicle ($n=6$) for 4 consecutive weeks starting 2 weeks after ET-1 first dosing. Values reported at each time point are the average of 3 consecutive measurements. Data are reported as mean ± SEM. $^{*}P<0.05$ versus respective vehicle group, *t*-test multiple comparisons. ERG, electroretinogram.

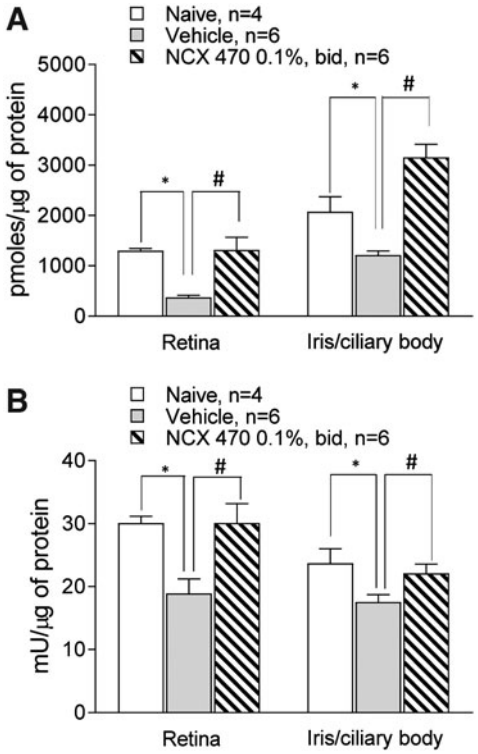

FIG. 4. Oxidative stress markers in retina and iris/ciliary body after NCX 470 repeated dosing following ET-1-induced ischemia/reperfusion injury in NZW rabbits. **(A)** GSH levels, and **(B)** MnSOD activity in dissected retina and iris/ciliary body of eyes that received 2-weeks ET-1 dosing followed by concomitant dosing with either NCX 470 (0.1%, bid) or vehicle for 4 additional weeks. Data are reported as mean ± SEM. $^{*}P<0.05$ versus naive group; $^{\#}P<0.05$ versus vehicle, *t*-test multiple comparisons. GSH, glutathione; MnSOD, manganese superoxide dismutase.

authors reported that high NO levels consequent to inducible nitric oxide synthase activation result in peroxynitrite formation and apoptotic cell death[36,37] others reported on the neuroprotective effects of NO signaling pathway stimulation in the eye as in other districts of the body.[38]

Due to technical limitation, the amount of NO released from NCX 470 could not be measured in our study, however, compounds having similar NO-donating moiety as that of NCX 470 ameliorate blood oxygenation in monkeys[39] and inhibit oxidative stress markers[22] following ET-1 induced ischemia/reperfusion. In the work reported here, we noted reduced levels of GSH and low MnSOD activity in ET-1-treated eyes that are reversed in NCX 470-treated eyes. These effects are likely dependent on NO release from NCX 470 and suggest that a reduction in oxidative stress possibly contributes to the beneficial effects of this compound.

Taken together, the results of this study would support NCX 470's having therapeutic properties over and above its clinically demonstrated IOP-lowering effects including retinal cell protective activity.

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2

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