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Title:
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Date:
2018-08-01

Citation:
Appukuttan, B., Ma, Y., Stempel, A., Ashander, L. M., Deliyanti, D., Wilkinson-Berka, J. L. & Smith, J. R. (2018). Effect of NADPH oxidase 1 and 4 blockade in activated human retinal endothelial cells. Clinical and Experimental Ophthalmology, 46 (6), pp.652-660. https://doi.org/10.1111/ceo.13155.

Persistent Link:
https://hdl.handle.net/11343/285030

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Original Article – Laboratory Science

Effect of NADPH oxidase 1 and 4 blockade in activated human retinal endothelial cells

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Short running title: Nox1/Nox4 blockade in retinal endothelial cells
Received 10 September 2017; accepted 10 January 2018
Conflict of interest: None
Funding sources: This work was supported by grants from the National Health & Medical Research Council (APP1078442), the Australian Research Council (FT130101648) and the Juvenile Diabetes Research Foundation (3-PDF-2017-376-AN)

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/ceo.13155

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ABSTRACT

Background: Over-production of reactive oxygen species (ROS) and resulting oxidative stress contribute to retinal damage in vascular diseases that include diabetic retinopathy, retinopathy of prematurity and major retinal vessel occlusions. NADPH oxidase (Nox) proteins are professional ROS-generating enzymes, and therapeutic targeting in these diseases has strong appeal. Pharmacological inhibition of Nox4 reduces the severity of experimental retinal vasculopathy. We investigated the potential application of this drug approach in humans.

Methods: Differential Nox enzyme expression was studied by RT-qPCR in primary human retinal endothelial cell isolates and a characterized human retinal endothelial cell line. Oxidative stress was triggered chemically in endothelial cells, by treatment with dimethyloxalylglycine (DMOG, 100 μM); Nox4 and VEGF transcript were measured, and production of ROS was detected by 2′,7′-dichlorofluorescein. DMOG-stimulated endothelial cells were treated with two Nox1/Nox4 inhibitors, GKT136901 and GKT137831; cell growth was monitored by DNA quantification, in addition to VEGF transcript and ROS production.

Results: Nox4 (isoform Nox4A) was the predominant Nox enzyme expressed by human retinal endothelial cells. Treatment with DMOG significantly increased endothelial cell expression of Nox4 over 72 hours, accompanied by ROS production and increased VEGF expression. Treatment with GKT136901 or GKT137831 significantly reduced DMOG-induced ROS production and VEGF expression by endothelial cells, and the inhibitory effect of DMOG on cell growth.

Conclusions: Our findings in experiments on activated human retinal endothelial cells provide translational corroboration of studies in experimental models of retinal vasculopathy and support the therapeutic application of Nox4 inhibition by GKT136901 and GKT137831 in patients with retinal vascular diseases.

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Key words: human; retina; endothelial cell; NADPH oxidase; vascular disease

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INTRODUCTION

Retinal vascular endothelial cell dysfunction and loss are key elements in the pathology that characterizes diabetic retinopathy, retinopathy of prematurity and major retinal vessel occlusions.[1-3] Accordingly, there is considerable interest in the therapeutic application of biologic drugs that limit endothelial cell damage in these retinal vasculopathies.[4-8] Multiple molecular changes are observed in the diseased retinal microvasculature; changes include the over-production of reactive oxygen species (ROS), and the resulting oxidative stress both contributes to and perpetuates the vascular disease.[1-3] High levels of ROS are associated with up-regulation of vascular endothelial growth factor (VEGF), which is a master regulator of retinal vascular leakage and neovascularization. Thus, the first biologic drugs to be applied successfully to the treatment of retinal vasculopathy block the action of VEGF.[8-10] Other biologically-targeted pharmaceuticals are at various stages in the development pipeline, including drugs that directly target the production of ROS.[11]

Reactive oxygen species include radicals and non-radicals: superoxide, hydrogen peroxide, hydroxyl radical, peroxynitrite, and hypochlorous acid. These molecules act deleteriously through the modification of a wide range macromolecules, but they also play physiological roles in cell signalling and have beneficial anti-microbial activity.[12,13] The NADPH oxidase (Nox) family of proteins have been termed "professional ROS-generating enzymes" in recognition of enzymatic activity dedicated to the production of ROS.[12] The family includes seven enzymes, which vary in structure, regulation, product and tissue distribution: Nox1 to Nox5, and dual oxidases (Duox), Duox1 and Duox2. One member – Nox4 – exists as two major isoforms, Nox4A and Nox4B.[14] Given the significant role of the Nox family members in the production of ROS, therapeutic targeting of these enzymes in retinal vasculopathy has strong appeal.

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Independent studies evaluating the expression of the five Nox enzymes, Nox1 through Nox5, in the same commercially-sourced human retinal endothelial cells, have identified Nox4 as the predominant isoform in this cell population.[15,16] Consistently, selective Nox blockade with a Nox1/Nox4 inhibitor reduces the production of ROS and the severity of neovascularisation in rat pups exposed to hyperoxia.[17,18] We sought to extend the potential application of this drug approach to the human. We first examined the expression of all seven Nox family enzymes in retinal endothelial cell isolates from multiple human donors, as well as a characterised human retinal endothelial cell line. We then evaluated the effect of two selective Nox1/Nox4 inhibitors on human retinal endothelial cell production of ROS, expression of VEGF, and growth. Oxidative stress was triggered chemically in these studies, by treatment with the prolyl-4-hydroxlase inhibitor, dimethyloxalylglycine (DMOG).

METHODS

Chemicals and drugs

Dimethyloxalylglycine was purchased from Sigma-Aldrich (St. Louis, MO) and used at a concentration of 100 μM, which was titrated in pilot studies to reliably induce the production of ROS. Two pyrazolopyridine dione drugs (i.e. GKT136901 and GKT137831) were provided by Genkyotex SA (Geneva, Switzerland). These drugs are well-characterised dual inhibitors of both Nox1 and Nox4 proteins, and they were identified in high throughput drug screens that involved cell-free assays of ROS production in human Nox4 membranes.[19-21] Drug design, synthesis and optimisation have been reported by the pharmaceutical manufacturer.[22,23] 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate, acetyl ester (CM-H_2DCFDA)

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and CyQUANT NF dye reagent were purchased from Thermo Fisher Scientific-Molecular Probes (Eugene, OR).

Endothelial cell isolation and culture

Endothelial cells were isolated from human retina, as we have previously described in detail.[24] In summary, paired retinae were digested with 0.25 mg-1 mg/ml Type II collagenase (ThermoFisher Scientific-GIBCO, Grand Island, NY), and cultured at 37 °C with 5% CO_2 in air, in MCDB-131 medium (Sigma-Aldrich) supplemented with 2% fetal bovine serum (FBS) (HyClone-GE Healthcare Life Sciences, Logan, UT) and endothelial growth factors (EGM-2 SingleQuots supplement; Clonetics-Lonza, Walkersville, MD). The FBS, hydrocortisone and gentamicin were omitted from the EGM-2 SingleQuots supplement. After 8 to 10 days, retinal endothelial cells were purified using magnetic Dynabeads (ThermoFisher Scientific-Invitrogen Dynal, Oslo, Norway) coated with mouse anti-human CD31 antibody (BD Biosciences-Pharmingen, San Diego, CA), and grown in modified MCDB-131 medium with 10% FBS. Retinal endothelial cells used in the experiments involving treatment with DMOG were previously transduced with the mouse recombinant amphotropic retrovirus, LXSN16E6E7,[25] to provide the needed numbers of cells for those studies. This cell line retains an endothelial phenotype, as we have previously reported.[24] The use of human cadaver donor eyes from the Eye Bank of South Australia (Adelaide, Australia), as a source of primary human retinal endothelial cell isolates for these studies, was approved by the Southern Adelaide Clinical Human Research Ethics Committee. Human cadaver eye donors ranged from 35 to 59 years at death, and time from death to processing of the retina averaged 17 hours.

RNA isolation and reverse transcription

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Cells were treated with Buffer RLT (Qiagen, Hilden, Germany) or Lysis Solution (Sigma-Aldrich, St. Louis, MO) with β-mercaptoethanol (Sigma-Aldrich), and total RNA was extracted using the RNeasy mini kit (Qiagen) or GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), according to the manufacturer's instructions and including the optional on-column DNase treatment. RNA concentration was determined by spectrophotometry on the NanoDrop 2000 instrument (Thermofisher Scientific, Wilmington, DE). Reverse transcription was performed using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA), with 500 ng (qPCR study of Nox isoform expression) or 100 ng (other PCR studies) of RNA template yielding 200 μL cDNA.

Quantitative real-time polymerase chain reaction

Quantitative real-time PCR (qPCR) was performed on the CFX Connect Real-Time PCR Detection System (Bio-Rad) using 2 μL of cDNA, 4 μL of iQ SYBRGreen Supermix, 1.5 μL each of 20 uM forward and reverse primers (Table 1), and 11 μL of nuclease-free water for each reaction. For the qPCR study of Nox isoform expression, amplification consisted of: a pre-cycling hold at 95 °C for 5 minutes; 40 cycles of denaturation for 30 seconds at 95 °C; annealing for 30 seconds at 58 °C; extension for 45 seconds at 72 °C; and a post-extension hold at 72 °C for 1 second. For other qPCR studies, amplification consisted of: a pre-cycling hold at 95 °C for 5 minutes; 40 cycles of denaturation for 30 seconds at 95 °C; annealing for 30 seconds at 60 °C; extension for 30 seconds at 72 °C; and a post-extension hold at 75 °C for 1 second. A melting curve was generated from 1-second holds at every 0.5 °C between 70 °C and 95 °C, to confirm that a single PCR product was produced. For the qPCR study of Nox isoform expression, PCR products were verified on 3% agarose gel with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific-Invitrogen, Carlsbad, CA).

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Reactive oxygen species detection assay

Human retinal endothelial cells were seeded for confluence in wells of black-wall and transparent-bottom 96-well microplates (Corning, Kennebunk, ME) in modified MCDB-131 medium with 10% FBS at 37 °C with 5% CO_2 in air.

Table 1: Primer pairs and product sizes for gene transcripts studied in human retinal endothelial cells.

Gene Transcript†	Primer Pair	Product Size (bp)
Nox 1	Forward: 5′-ACCGGTCATTCTTTATATCTGTGAAA-3′ Reverse: 5′-GTTCATCTGCAATTCCAAAACTTTG-3′	115
Nox2	Forward: 5′-TGGCATGGATGATTGCACTTC-3′ Reverse: 5′-TGACTCGGGCATTCACACA-3′	84
Nox3	Forward: 5′-CTATGGGAGGCCCAACTGGAA-3′ Reverse: 5′-AACTGGGAGCTCATATCAACAG-3′	288
Nox4	Forward: 5′-CCGGCTGCATCAGTCTTAACC-3′ Reverse: 5′-TCGGCACAGTACAGGCACAA-3′	220
Nox4A	Forward: 5′-GTGGCTGGAGGCATTGGAGT-3′ Reverse: 5′-TGCAAACCAACGGAAGGACTG-3′	125
Nox4B	Forward: 5′-AGAATTTATCCCAAGGATGACTGGA-3′ Reverse: 5′-TGCAAACCAACGGAAGGACTG-3′	96
Nox5	Forward: 5′-CCCACCATTGCTCGCTATG-3′ Reverse: 5′-CCGAATGTGCAGCCAGATAGT-3′	84
Duox1	Forward: 5′-GGACCGGACTCACTTCTCCCA-3′ Reverse: 5′-CTACTTCTCACTCTAGTCCCT-3′	278
Duox2	Forward: 5′-TTCACGCAGCTCTGTGTCAA-3′ Reverse: 5′-TGATGAACGAGACTCGACAGCT-3′	94
RPLP0	Forward: 5′-GCAGCATCTACAACCCTGAA-3′ Reverse: 5′-GCAGATGGATCAGCCAAGAA-3′	235
VEGFA	Forward: 5′-TGCTGTCTTGGGTGCATT-3′ Reverse: 5′-GTGCTGTAGGAAGCTCATCTC-3′	365

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YWHAZ	Forward: 5′-ACTTTTGGTACATTGTGGCTTCAA-3′ Reverse: 5′- CCGCCAGGACAAACCAGTAT-3′	98

✝Abbreviations: RPLP0 = ribosomal protein lateral stalk subunit P0; VEGFA = vascular endothelial growth factor A; YWHAZ = tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

On the following day, the medium was replaced with fresh medium that either contained or did not contain DMOG. In some experiments, the cells were also treated with 100 nM GKT136901 or GKT137831. Medium and supplements were changed every 24 hours for experiments that ran over 24 hours. At pre-determined time intervals, the assay was stopped by staining cells with 10 µM CM-H_2DCFDA, freshly diluted in phenol red-free EBM medium with 10% FBS (Clonetics-Lonza) for 15 minutes at 37 °C in the dark. The CM-H_2DCFDA was replaced with modified EBM medium ahead of reading on a fluorescence microplate reader (VICTOR X3, PerkinElmer, Singapore) with excitation and emission filters of 485 nm and 535 nm, respectively. In some experiments, wells were also photographed on the EVOS FL Cell Imaging System (Thermo Fisher Scientific-Invitrogen) at 10-times magnification.

Endothelial cell growth assay

Cells were plated and treated as described for the ROS detection assay. After 72 hours, cell growth was stopped by staining cells with CyQUANT NF dye reagent, freshly diluted in Hank's balanced salt solution for 1 hour at 37 °C in the dark. Subsequently, the CyQUANT NF dye reagent was replaced with phosphate buffered saline, and the wells were read on a fluorescence microplate reader with excitation and emission filters of 485 nm and 535 nm, respectively.

Statistical methods

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Data for test and control conditions were compared by two-tailed Student's t-tests for two-condition comparisons and one-way ANOVA tests with post-hoc Tukey's testing for multiple-condition comparisons, using CFX Manager v3.1 (Bio-Rad) or GraphPad Prism v6.04 (GraphPad Software, La Jolla, CA). In all analyses, a significant difference between conditions was defined as one yielding a *p*-value less than 0.05.

RESULTS

The pattern of expression of up to five Nox enzymes – Nox1, Nox2, Nox3, Nox4 and Nox5 – in a commercially available human retinal endothelial cell isolate has been reported by two independent groups.[15,16] Individual humans may demonstrate considerable differences in gene expression, and thus expression of the complete family of Nox enzymes, including Duox1 and Duox2, plus Nox4A and Nox4B isoforms, was investigated by RT-qPCR in human retinal endothelial cells separately isolated from 5 human donor eyes. Consistently across the 5 primary endothelial cell isolates, Nox4 was the predominantly expressed enzyme, with other enzymes expressed at negligible or relatively low levels (Figure 1A and 1B). Of the two Nox4 isoforms, Nox4A had approximately 10-fold higher expression than Nox4B. The same pattern of enzyme expression seen in the primary endothelial cell isolates was also measured in a characterised human retinal endothelial cell line (Figure 1A), which therefore was used for subsequent experiments.

Human retinal endothelial cells were stimulated with DMOG for up to 72 hours, and expression of Nox4 and angiogenic target, VEGFA, were measured by RT-qPCR.

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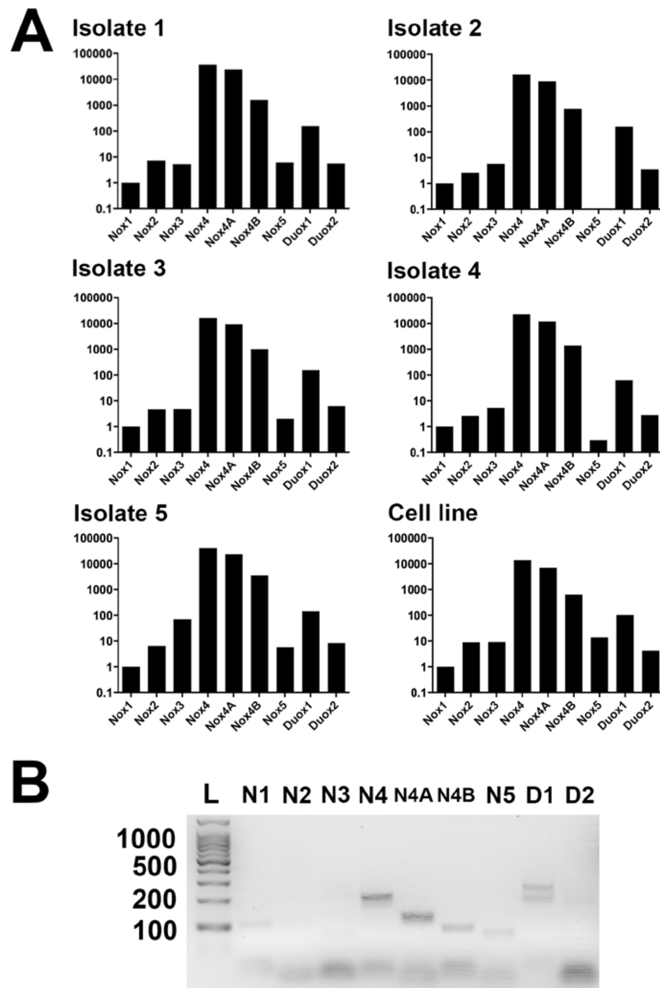

Figure 1: Relative expression of NADPH oxidase (Nox) family members in human retinal endothelial cells isolated from 5 human donor eye pairs (Isolates 1-5) and one human retinal endothelial cell line (Cell line). (A) Graphs show normalized transcript expression for each enzyme. Reference gene was ribosomal protein lateral

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stalk subunit P0. Data are expressed as fold-change relative to Nox1. (B) Representative gel image (corresponding to Isolate 1) showing qPCR products run on 3% agarose gel. Abbreviations: L = DNA ladder; N = Nox; D = Duox. Expected products sizes: Nox1 = 115 bp; Nox2 = 84 bp; Nox3 = 288 bp; Nox4 = 220 bp; Nox4A =125 bp; Nox4B = 96 bp; Nox5 = 84 bp; Duox1 = 278 bp (upper band); Doux2 = 94 bp. Bands at lower edge of gel represent primer dimer products. Controls generated with no template did not amplify.

Cellular expression of VEGFA was determined as transcript, not protein, because MCDB-131 medium is supplemented with human recombinant VEGF, which would confound the results of a protein assay. Production of ROS was measured in parallel, using CM-H_2DCFDA as an indicator: intracellular oxidation of the processed molecule yields a fluorescent product. Expression of Nox4 increased significantly in DMOG-exposed endothelial cells in comparison to expression in control cells treated with medium alone at 24, 48 and 72 hours of stimulation (Figure 2A). Imaging after CM-H_2DCFDA treatment demonstrated increased fluorescence of cell monolayers exposed to DMOG versus control monolayers across all time points, but most obviously at 48 and 72 hours (Figure 2B). Measurements of cell fluorescence by plate reader consistently indicated increased levels in DMOG-exposed cells, which were significantly higher than measurements of control cells at 48 and 72 hours (Figure 2C). The DMOG-stimulated cells also significantly increased the expression of VEGFA from baseline levels at 24, 48 and 72 hours (Figure 2D). These results indicate DMOG induces production of ROS and VEGF in human retinal endothelial cells.

There is no drug currently prescribed in humans that inhibits Nox4 alone. However, the pyrazolopyridine dione drugs, GKT136901 and GKT137831, are selective Nox1/Nox4 inhibitors.[21] Having demonstrated that Nox4 was expressed by human

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retinal endothelial cells and that increased expression, induced by DMOG, was associated with the production of ROS and VEGFA by the cells, we investigated the effect of the Nox1/Nox4 inhibitors on these Nox4-related activities. Similar effects were observed following treatment with GKT136901 or

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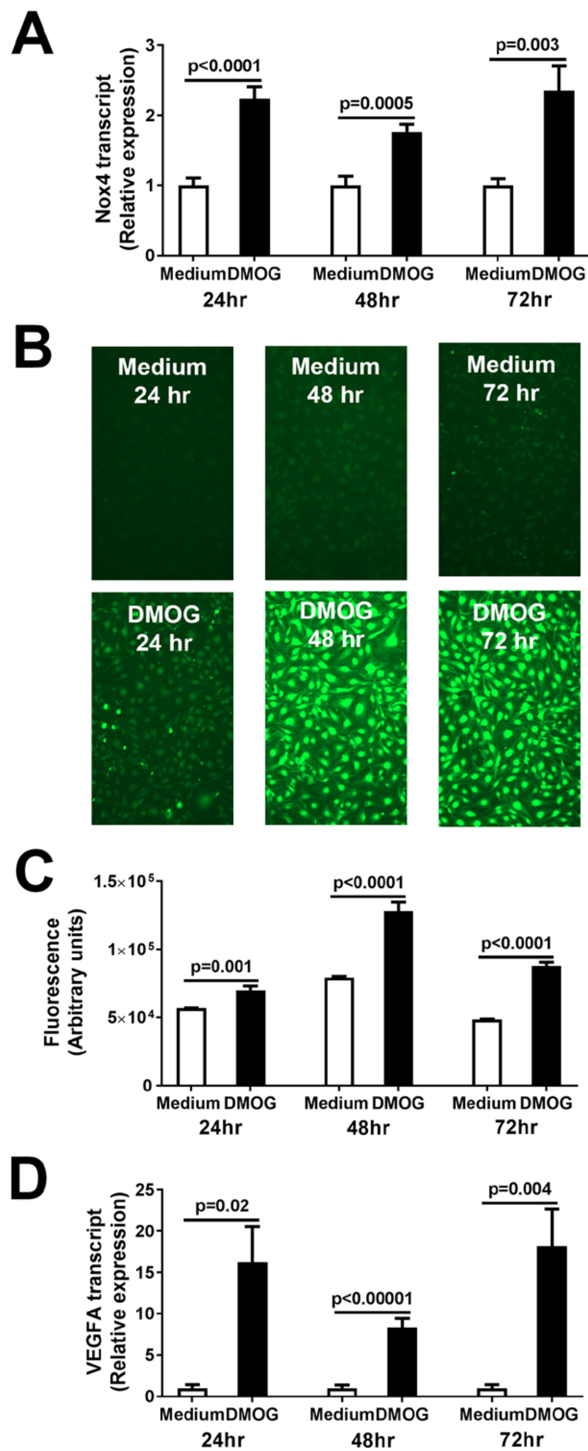

Figure 2: Effects of stimulation of human retinal endothelial cells with dimethyloxalylglycine (DMOG) (evaluated time points post-stimulation = 24, 48 and

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72 hours). Graphs showing relative expression of (A) Nox4 and (D) vascular endothelial growth factor (VEGF)A transcripts, and (C) relative production of reactive oxygen species (ROS) in DMOG-exposed versus control medium-treated retinal endothelial cells. Reference genes for RT-qPCR (A and D) were ribosomal protein lateral stalk subunit P0 and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta. Bars represent mean relative expression, with error bars showing standard error of mean. n = 6 (A and D) or n = 8 (C) cultures/condition. Data were analysed by two-tailed Student's t-test. (B) Photomicrographs of DMOG-exposed versus medium control-treated retinal endothelial cells incubated with CM-H_2DCFDA to detect ROS (green). Original magnification: 10X.

GKT137831: both drugs significantly reduced production of ROS (Figures 3A and 3B) and expression of VEGFA (Figures 3C and 3D) by DMOG-stimulated human retinal endothelial cells. In an independent experiment, neither drug altered the constitutive level of VEGFA expression (Supplementary Figure 1).

A key feature of retinal vasculopathy is loss of endothelial cells from the retinal vasculature.[1,26] Therefore, we also examined the effect of the Nox1/Nox 4 inhibitors on retinal endothelial cell growth, using an assay in which final cell number was estimated from binding of fluorescent dye to cellular DNA. In this assay, 72-hour treatment with DMOG significantly reduced fluorescence of retinal endothelial cell monolayers from baseline, indicating reduced cell proliferation (Figures 4A and 4B). Treatment with GKT136901 or GKT137831 did not impact cell growth in comparison to that of control cell monolayers treated with medium alone, but resulted in significant reversal of the inhibitory effect of DMOG. These results indicate that selective Nox4 inhibition with GKT136901 or GKT137831 limits the generation of ROS and VEGFA, and promotes normal cell growth under conditions of oxidative stress.

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DISCUSSION

The seven members of the Nox family of enzymes are differentially expressed by different cell populations in different body tissues. We have found Nox4 – and its A isoform in particular – to be the predominantly expressed enzyme in human retinal endothelial cell isolates from 5 different human donors, as well as a human retinal endothelial cell line. This observation confirms and extends previous investigations. Studying human retinal endothelial cells from the same commercial source (Cell Systems; Kirkland, WA), Li et al.[15] identified Nox4 as predominant over Nox1 and Nox2, and Wang et al.[16] identified Nox4 as predominant over Nox1, Nox2, Nox3 and Nox5. We have highlighted differences in retinal endothelial cell gene expression between individuals.[27] For the Nox enzymes, however, we saw a remarkably similar pattern of expression across multiple cell isolates. Nox4 is also the predominant Nox enzyme expressed in other human vascular endothelial populations, including aortic, pulmonary arterial, umbilical venous and coronary arterial endothelial cells.[28-31]

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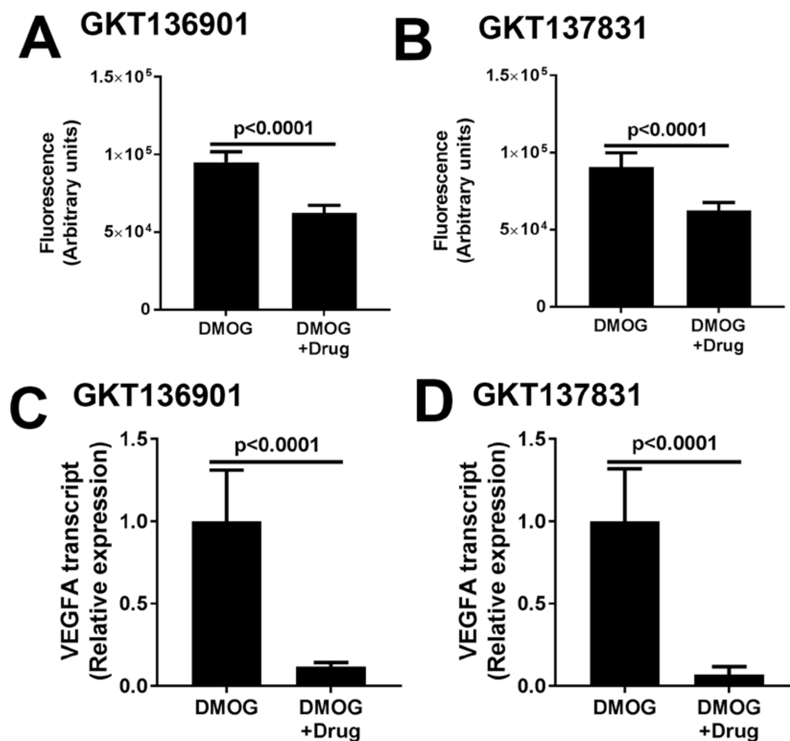

Figure 3: Effects of GKT136901 and GKT137831 on production of reactive oxygen species (ROS) and expression of vascular endothelial growth factor (VEGF)A transcript by human retinal endothelial cells following treatment with dimethyloxalylglycine (DMOG) (evaluated time point post-stimulation = 72 hours). Graphs showing (A and B) relative production of ROS and (C and D) relative

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expression of VEGFA transcript in DMOG-exposed retinal endothelial cells, (A and C) treated with GKT136901 or not treated, and (B and D) treated with GKT136901 or not treated. Reference genes for RT-qPCR (A and D) were ribosomal protein lateral stalk subunit P0 and tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein zeta. Bars represent mean relative expression, with error bars showing standard error of mean. n = 8 (A and B) or n = 5 (C and D) cultures/condition. Data were analysed by two-tailed Student's t-test.

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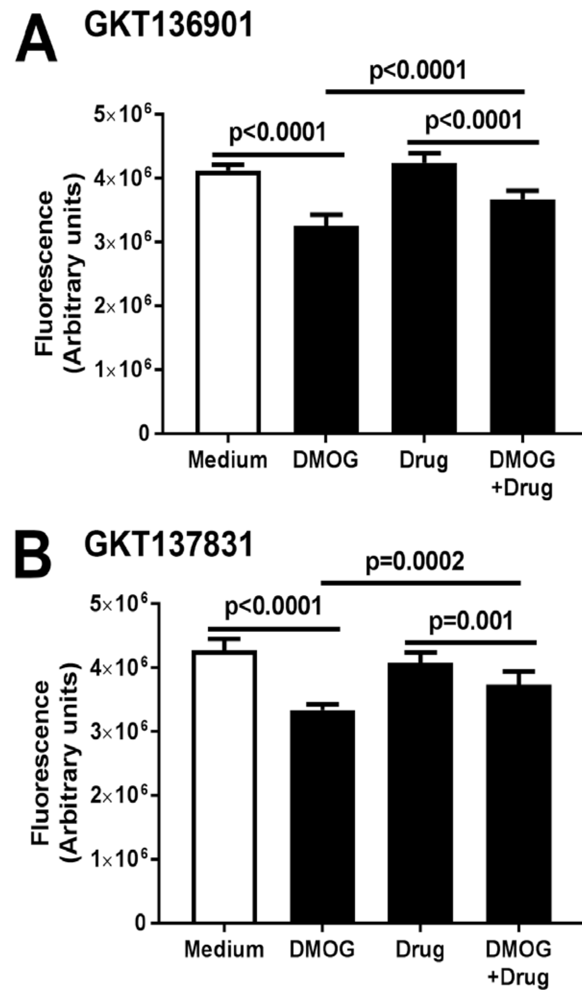

Figure 4: Effects of GKT136901 and GKT137831 on human retinal endothelial cell growth following treatment with dimethyloxalylglycine (DMOG) (evaluated time point post-stimulation = 72 hours). Graphs of relative cell number in DMOG- versus

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control-exposed retinal endothelial cells, (A) treated with GKT136901 or not treated, and (B) treated with GKT136901 or not treated. Bars represent mean relative expression, with error bars showing standard error of mean. n = 8 cultures/condition. Data were analysed by one-way ANOVA tests with post-hoc Tukey's testing. Results of Tukey's testing are shown on graphs.

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As was comprehensively summarised in the recent review by Wang and Hartnett,[32] Nox4 participates in endothelial homeostatic and pathological processes; the nature of this involvement relates to multiple variables that include tissue location and timing, as well as the process in question. Thus, in comparison to control animals, Nox4 gene-deleted mice or mice treated with Nox4 inhibitor demonstrate: reduced capillary growth in the gastrocnemius muscle, in response to running exercise on a wheel or treadmill;[33] poor recovery of capillary density and blood flow in the leg following femoral artery ligation;[34] and less aggressive pulmonary neovascularisation in experimental pulmonary hypertension.[35] In the mouse eye, Nox4 deficiency does not impact the normal radial outgrowth of retinal blood vessels from the optic nerve in the developing retina.[33] However, Nox4 has been identified as a mediator of mouse and rat vascular retinopathies, in studies conducted by several independent groups.[15,16,33,36]

Our work – involving exposure of human retinal endothelial cells to DMOG – is more relevant to retinal pathology than homeostasis. Within 24 hours of treatment with DMOG, and across the 72-hour duration of the exposure, endothelial cells significantly increased their expression of Nox4. Up-regulation of the oxidase was associated with augmented production of ROS and expression of VEGF, which are key players in the development of retinal vasculopathy. Diabetic leptin receptor^{db} homozygous mice experience increased expression of Nox4 in the retina,[15,37] and intravitreal delivery of a Nox4-targeted small interfering RNA reduces retinal VEGFA and retinal vascular leakage, in parallel with reduced Nox4 expression.[15] Retinal Nox4 expression is also increased in the mouse model of oxygen-induced retinopathy,[16,36] and Nox4 blockade through genetic deletion or RNA interference reduces pathological retinal neovascularisation in this model.[33,36]

Given constitutive and inducible expression of Nox4 in human retinal endothelial cells, plus the protective effect of Nox4 genetic deletion or RNA interference in

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experimental retinal vascular disease, there is clear potential for Nox4 blockade as a treatment of retinal vasculopathies, including diabetic retinopathy. GKT136901 and GKT137831 are pyrazolopyridine diones that target Nox1 and Nox4.[21] Studies of these two drugs in mouse models of diabetic nephropathy have showed effects that include preservation of renal architecture and reduced albuminuria.[38,39] GKT137831 has already been administered to in human subjects: the drug was well tolerated by patients in one short phase II trial for diabetic nephropathy, and a 48-week phase II study in patients with type I diabetes mellitus and early nephropathy was recently launched in Australia. In two reports, Wilkinson-Berka et al.[17,18] have described beneficial effects of GKT137831 in rat oxygen-induced retinopathy, including reduced retinal vascular leakage, avascularity and neovascularisation.

Our experiments indicate both GKT136901 and GKT137831 reduce DMOG-induced production of ROS and expression of VEGFA by human retinal endothelial cells. A central early event in retinal vasculopathy is endothelial cell loss,[26,40] and importantly, GKT136901 and GKT137831 also significantly reverse the negative effect of DMOG on human retinal endothelial cell growth. The DMOG-associated reduction in cell growth likely reflects a negative effect on cell cycle progression. Hypoxia-inducible factor (HIF)-1α induces cell cycle arrest.[41] In keeping with the HIF-1α-stabilizing activity of DMOG, we have measured reduced expression of cell cycle promoter, cyclin D1, in human retinal endothelial cells treated for 72 hours with DMOG; consistently, we have observed that GKT136901 and GKT137831 prevent this reduction in cyclin D1 expression (data not shown). However, given that Nox1/Nox4 inhibition only partially reverses the negative effect of DMOG on cell growth, signalling pathways other than Nox4-mediated ROS production may contribute to the growth inhibition associated with DMOG treatment. Alternatively, or additionally, technical factors such as the concentration or timing of application of the Nox1/Nox4 inhibitors also may impact the degree of reversal.

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In summary, our studies in human retinal endothelial cells provide a translational corroboration of studies conducted in rodents that support the therapeutic potential for targeted Nox inhibition with GKT137831 in patients with retinal vasculopathy. Nox4 is the predominant Nox enzyme expressed by human retinal endothelial cells, and inhibition of enzyme induction limits production of ROS by these cells and promotes their growth. Our work also suggests that possibility of targeting Nox4 in other retinal diseases that involve oxidative stress in the retinal endothelium, including proliferative vitreoretinopathy and posterior uveitis.[42,43]

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Acknowledgements

The authors wish to thank Denise A. Galloway, PhD (Fred Hutchinson Cancer Institute Seattle, WA) for the gift of the LXSN16E6E7 virus.

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