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Sodium Orthovanadate Effect on Outflow Facility and Intraocular Pressure in Live Monkeys

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Abstract

Sodium orthovanadate (Na_3VO_4) is reported to reduce IOP by affecting aqueous formation, but whether it also affects outflow facility (OF) is unclear. We tested the effect of Na_3VO_4 on OF and intraocular pressure (IOP) in live cynomolgus monkeys, and on actin and cell adhesion organization in cultured human trabecular meshwork (HTM) cells. Total OF ($n = 12$) was measured by 2-level constant pressure perfusion of the monkey anterior chamber (AC) before and after exchange with 1 mM Na_3VO_4 or vehicle in opposite eyes. Topical 1% Na_3VO_4 or vehicle only was given twice daily (each $2 \times 20 \mu\text{L}$ drops) for 4 days to opposite eyes ($n = 8$), and Goldmann IOP was measured before and hourly after treatment for 6 hours on Days 1 and 4. Filamentous actin and vinculin-containing cell adhesions were examined by epifluorescence microscopy after the cells had been incubated with 1 mM Na_3VO_4 for 24 hours. A) In monkeys, Na_3VO_4 increased OF by $29.3 \pm 8.8\%$ (mean $\pm$ s.e.m.) over the perfusion interval when adjusted for baseline and contralateral eye washout ($p = 0.01$; $n = 12$). B) Day 1 baseline IOP was 16.2 ± 1.5 mmHg in treated eyes and 15.9 ± 1.3 mmHg in the contralateral control eyes. Following treatment on Day 1, IOP was no different ($p > 0.05$) between treated eyes and control eyes at any time-point or compared to baseline. Day 4 mean IOP averaged over hours 2–6 was 13.5 ± 0.8 mmHg in treated eyes and 16.1 ± 0.2 mmHg in control eyes. Treated eye IOP was lower than its Day 4 baseline ($p < 0.005$), lower than control eyes for the same Day 4 interval ($p = 0.009$), and lower than the Day 1 baseline ($p = 0.0000$). Control eye IOP on Day 4 was not significantly different from baseline on Day 1. C) Incubation of HTM cells with 1 mM Na_3VO_4 for 24 hours caused a loss of actin stress fibers and vinculin-containing adhesions. Cell retraction and separation was also observed in vanadate-treated cultures. Reformation of actin stress fibers, vinculin-containing adhesions and confluent monolayers occurred within 24 hours after Na_3VO_4 -containing culture medium was replaced with Na_3VO_4 -free medium. Ocular administration of Na_3VO_4 to live monkeys significantly increases OF and reduces IOP. Na_3VO_4 reversibly disrupts actin and cell adhesion organization and causes retraction and separation of cultured HTM cells.

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Na₃VO₄ increases pressure-dependent outflow in live monkeys. Altered actin architecture in the TM may play a part in this increased OF.

Keywords

cytoskeleton; trabecular meshwork; aqueous humor outflow; phosphorylation; intraocular pressure

INTRODUCTION

Vanadate lowers intraocular pressure (IOP) when it is administered topically to live rabbit eyes (Krupin et al., 1980) and monkey eyes with normal IOP (Podos et al., 1984) or experimentally elevated IOP (Becker, 1980; Lee et al., 1987). The reduced IOP from vanadate has been attributed to Na⁺/K⁺-ATPase inhibition and decreased aqueous humor formation, but not to altered outflow facility (Becker, 1980; Mittag et al., 1984).

There is reason to suspect, however, that vanadate can increase the fluid conductivity of the outflow pathway. In addition to inhibiting Na⁺/K⁺-ATPase, vanadate can alter the actin cytoskeleton and cell-matrix adhesions. For example, in cultured bovine aortic endothelial cells vanadate induces disassembly of actin microfilaments and cell adhesions (Ayalon and Geiger, 1997). It is not known if vanadate has a similar effect on the actin-cell adhesion organization of trabecular meshwork (TM) cells. This is relevant as the state of TM actomyosin contractility is an important determinant of aqueous outflow physiology (Rao et al., 2005; Tian et al., 2009; Tian and Kaufman, 2005).

We have sought to examine the possibility that the vanadate salt, sodium orthovanadate (Na₃VO₄), affects outflow facility in live non-human primates. Unlike previous studies that used the indirect method of tonography (Becker, 1980; Mittag et al., 1984), we perfused the anterior chambers in live monkey eyes. Separately, IOP measurements were performed after administering topical Na₃VO₄ to live monkey eyes. We then tested the effect of Na₃VO₄ on the actin cytoskeleton and vinculin-containing cell adhesions of cultured human TM (HTM) cells.

MATERIAL AND METHODS

Sodium orthovanadate preparation

For perfusion experiments in live monkey eyes, 10 mM Na₃VO₄ was dissolved in water as a stock solution and adjusted to pH10 with NaOH at 100°C. Na₃VO₄ solution was diluted to final concentrations of 500 μM and 1 mM in Bárány's mock aqueous humor solution (Bárány, 1964) adjusted to pH 7.4 with HCl, and cooled just before being used for perfusion experiments to determine outflow facility.

For topical application to live monkey eyes, 1% Na₃VO₄ was dissolved in 10% DMSO in water with 5% Tween-20. The pH was adjusted to 10 with NaOH and boiled for 5 min. Once dissolved, the solution was cooled at room temperature and adjusted to pH 7.4 with HCl.

For tissue culture studies, 10 mM Na₃VO₄ (Sigma-Aldrich Corp, St Louis, Mo) was dissolved in water as a stock solution, adjusted to pH10 with NaOH, and boiled at 100°C for 5 min. The pH of the solution was then readjusted to 7.4 with HCl while stirring to avoid precipitation of Na₃VO₄. The Na₃VO₄ solution was diluted to a final concentration of 1 mM in Dulbecco's Modified Eagle's Medium (DME; Sigma-Aldrich Co., St. Louis, MO) before being added to cultured HTM cells.

Animals, Sedation and Anesthesia

Seventeen adult cynomolgus monkeys (*Macaca fascicularis*) of both sexes weighing 2.6–7.8 kg with no ocular abnormalities were studied. Three of the monkeys were used in both IOP and outflow facility experiments, with at least 6 months elapsing between experiments. Monkeys received I.M. ketamine (10 mg/kg initially at baseline, then 5 mg/kg supplements with IOP measurements) to measure IOP and for eye drop administration. Anesthesia for anterior chamber (AC) perfusion to measure outflow facility was induced with I.M. ketamine followed by I.V. pentobarbital Na (15 mg/kg initial; 5–10mg/kg supplemental). All experiments were done in accordance with the ARVO statement on the Use of Animals in Ophthalmic and Vision Research.

Live Monkey Protocol 1: Effect of 1 mM Sodium Orthovanadate on Outflow Facility

Total outflow facility was measured in 12 monkeys by 2-level constant pressure perfusion (2.5 and 11.9 mmHg above spontaneous IOP) of the anterior chamber with Bárány's solution (Bárány, 1964). The AC of each eye was cannulated with one branched and one unbranched 26-gauge needle. One end of the branched needle was attached to an elevated reservoir containing Bárány's solution (Bárány, 1964) and the other to a pressure transducer. Tubing to the non-branched needle was clamped. Baseline outflow facility was measured for 35–40 min. The tubing from the non-branched needle was then attached to a variable speed infusion pump with the inflow line of the branched needle detached from the reservoir and open to air.

The AC of one eye was exchanged with 2 mL of 1 mM Na_3VO_4 ; the opposite eye was exchanged with 2 mL vehicle (90% Bárány's + 10% water by volume). The tubing from the non-branched needle was re-clamped and reservoirs reconnected, but closed to the eye for 60–90 min to allow for stabilization and maximum drug effect for both the 100 μM and 1 mM doses. The reservoirs were then opened and facility determined for 60–90 min with infusion of corresponding drug/vehicle solution into the eyes. In 5 monkeys, the 1 mM Na_3VO_4 exchange was preceded by AC exchange with 100 μM Na_3VO_4 in the treated eye or vehicle in the control eye and outflow facility measured for ~ 45 min.

Live Monkey Protocol 2: Effect of 1% Sodium Orthovanadate on Intraocular Pressure

Topical 1% Na_3VO_4 (2×20 μL drops ~1 min apart) was instilled in one eye of 8 supine monkeys twice daily for 4d; vehicle (10% DMSO, 5% Tween 20 in ddH₂O) was given to the opposite eye. Proparacaine HCl (1×30–40 μL drop) was administered to both eyes prior to Na_3VO_4 /vehicle treatment to increase corneal permeability. IOP was measured by Goldmann applanation tonometry (Kaufman and Davis, 1980)¹² at baseline and at hours 0, 0.5, 1, 1.5, 2, 3, 4, 5, and 6 on Days 1 and 4 of treatment in 4 monkeys. In the 4 other animals IOP was measured at baseline and on Day 4 as above.

HTM Cell Culture and Epifluorescence Microscopy

Human trabecular meshwork (HTM) cells were isolated as previously described (Polansky et al., 1979, 1984). The cells were plated at 50% confluence and grown to confluence on glass cover slips in 24-well plates (Corning, Inc., Corning, NY). The cells were cultured in low-glucose Dulbecco's Modified Eagle's Medium (DME; Sigma-Aldrich Co., St. Louis, MO), 15% fetal bovine serum (FBS; Atlanta Biologicals, Inc, Norcross, GA), 2 mM L-glutamine, 2.5 $\mu\text{g}/\text{ml}$ amphotericin B, 25 $\mu\text{g}/\text{ml}$ gentamicin, and 1 ng/ml FGF-2 (PeproTech, Inc., Rocky Hill, NJ), as previously described (Filla et al, 2002; Polansky et al., 1979, 1984). Upon reaching confluence, media was exchanged daily. Seven days after HTM cells had reached confluence, FBS was reduced to 10%, FGF-2 was stopped, and experiments were performed.

The culture medium was replaced with serum-free DME medium containing 1 mM Na_3VO_4 prepared as described above. Treated HTM cells were incubated for 24 hours. The cells were washed with 50 mM MES (2-(*N*-morpholine) ethanesulfonic acid) buffer, permeabilized with 0.5% Triton X-100 (Sigma) in MES and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , and 1.5 mM KH_2PO_4). The cells were then incubated in 1% bovine serum albumin (BSA) in PBS to block non-specific labelling. Blocked cells were labelled with monoclonal anti-human vinculin antibody (Sigma-Aldrich) 1:3000 in 0.1% BSA/PBS for 1 h. The cells were washed and incubated simultaneously with Alexa 546-conjugated goat anti-mouse secondary antibody (4 $\mu\text{g}/\text{ml}$; Molecular Probes, Eugene, OR) and Alexa 488-conjugated phalloidin (0.67 unit/ml; Molecular Probes) in 0.1% BSA/PBS for 1 h. Coverslips were mounted onto slides (Immunomount; Shandon Lipshaw, Pittsburgh, PA), and images were acquired with a camera (AxioCam HRm; Carl Zeiss Meditec, Inc., Thornwood, NY) mounted on a fluorescence microscope (Axiophan 2 Imaging together with AxioVision version 3.1 software; Carl Zeiss Meditec, Inc.). To assess reversibility of the Na_3VO_4 effect, the media on cells incubated with Na_3VO_4 for 24 h was removed and fresh medium with 10% FBS was added for an additional 24 h. Cells were washed, fixed, labeled, and examined as described above.

RESULTS

Outflow Facility

Outflow facility following AC exchange with 1 mM Na_3VO_4 was $44 \pm 10\%$ ($p \leq 0.005$) higher in treated than contralateral control eyes (Table 1). Adjusted for their respective baselines and non-drug perfusion-related washout, outflow facility was $29.3 \pm 9.0\%$ ($p \leq 0.01$) higher in eyes treated with 1 mM Na_3VO_4 than controls ($n = 12$). Outflow facility tended to be higher ($26 \pm 10\%$), though not significantly ($p \leq 0.10$), in eyes exchanged with 100 μM Na_3VO_4 compared with contralateral controls after correcting for their respective baselines ($n = 5$).

Intraocular Pressure

On day 1 the mean baseline IOP in treated and control eyes was 16.2 ± 1.5 mm Hg and 15.9 ± 1.3 mm Hg, respectively (Fig. 1A). Following a single treatment with Na_3VO_4 , IOP was unchanged at any time-point on the first day. Day 4 mean IOP averaged over 2–6 hours (mean of $n = 5$ time points) was 13.5 ± 0.8 mm Hg in treated eyes and 16.1 ± 0.2 mm Hg in control eyes. Day 4 (Fig. 1B) treated-eye mean IOP averaged over hours 2–6 was lower than its Day 4 baseline ($p < 0.005$. Range of reduction: 0.44–3.56 mm Hg), lower than control eyes for the same Day 4 interval ($p = 0.009$. Range of reduction: 2.0–5.3 mm Hg), and lower than the Day 1 treated-eye baseline ($p = 0.0000$ Range of reduction: 3.0–6.5 mm Hg) (Fig. 1C). Day 4 control mean IOP was not significantly less than the Day 1 baseline (Fig. 1C). The Day 4 magnitude of IOP reduction in Na_3VO_4 -treated eyes compared with the Day 1 or Day 4 baselines was significantly more than in the corresponding controls for the mean of 5 time-points over the matching 2–6 hour treatment intervals ($p \leq 0.05$. Range of reduction: 3.0–6.5 mm Hg) (Fig. 1D). In subsequent IOP measurements in treated eyes weeks after treatment had ended, IOP was noted to have returned to the pre-treatment baseline.

Effect on HTM Cells in Culture

In untreated HTM cells (Fig. 2A), linear arrays of actin stress fibers were seen. Vinculin staining (Figs. 2C) was punctate and distributed around the periphery of the cells throughout HTM monolayers. Higher magnification indicated that vinculin was in focal adhesions at the tips of the actin stress fibers (Fig. 2E).

Following treatment with 1 mM Na_3VO_4 staining of F-actin was reduced (Fig. 2B). Actin stress fibers were rarely seen. In addition, gaps had formed between HTM cells indicating that the

cells had retracted and the monolayers were disrupted (Fig. 2B, D, F). Vinculin-stained cell adhesions were markedly reduced (Fig. 2D & F). When Na_3VO_4 -containing medium was removed and replaced with Na_3VO_4 -free medium with 10% FBS, the actin cytoskeleton (Fig. 2G), vinculin-stained adhesions (Fig. 2H) and confluent cell monolayers reformed over time indicating that the effect of Na_3VO_4 was reversible.

DISCUSSION

We have found that perfusing Na_3VO_4 into the anterior chamber of live non-human primate eyes enhances outflow facility. 1 mM Na_3VO_4 caused a significant 29% increase in outflow facility when adjusted for control washout and baseline measurements. IOP was decreased after topically administering 1% Na_3VO_4 . We studied the effect of vanadate on the actin cytoskeleton of TM cells *in vitro*. While this did not pinpoint the exact site of action of vanadate in outflow tissue, it provided clues to how vanadate could have affected outflow physiology. In cultured HTM cells, 1 mM Na_3VO_4 caused disassembly of actin microfilaments and vinculin-containing cell adhesions, cell retraction, cell-cell separation and a disruption of monolayers. That these findings were reversible raises the possibility that Na_3VO_4 affected regulatory components of the outflow tissue.

We designed our perfusion experiments to favor the action of vanadate on the outflow tissue and cells. Vanadate, VO_4^{3-} , is an ion with a tetrahedral structure, enabling it to act as a phosphate analogue and inhibit protein tyrosine phosphatases, alkaline phosphatases and ATPases such as Na^+/K^+ -ATPase (Gordon, 1991; Soulsby and Bennett, 2009). A typical application of Na_3VO_4 is to add it to lysis buffers to inhibit endogenous phosphatases and preserve the phosphorylation states of proteins during protein lysate incubation in a buffer. Our exchange perfusion sought to mimic this by exposing the outflow tissue of live monkeys to Na_3VO_4 over a fixed period before starting facility measurements. Likewise, cultured HTM cells were incubated in Na_3VO_4 -containing culture medium.

We found increased outflow facility after ocular administration of vanadate, whereas previous studies did not. While previous studies measured outflow facility indirectly by tonography (Becker, 1980; Mittag et al., 1984), we used anterior chamber perfusion methodology. Tonography introduces greater variability, assumptions and artifacts than the perfusion approach (Kaufman, 1996). We administered vanadate directly into the anterior chamber to ensure outflow tissue incubation at a known vanadate concentration. Previous studies administered vanadate topically (Becker, 1980; Mittag et al., 1984). Topically-administered agents are subject to a different kinetic of ocular penetration and access to the aqueous secretion and drainage tissues compared with anterior chamber perfusion. These differences in technique and pharmacokinetics may have been significant enough to account for failure of the previous studies to observe the modest Na_3VO_4 -induced outflow facility change that we report here.

Our main aim was to determine if Na_3VO_4 affects the fluid conductivity of the outflow tissue as might be predicted from an expected effect on actin and cell adhesion organization. Contrary to previous studies, we have evidence that Na_3VO_4 does increase outflow facility in live non-human primates. Vanadate can affect cells in many ways, and different cell types exist in the anterior chamber and outflow route that may have been affected by the treatment. In light of the known biological actions of Na_3VO_4 , our *in vivo* and *in vitro* findings suggest that altered phosphorylation activity related to vanadate inhibition of phosphotyrosine phosphatases, Na^+/K^+ -ATPase, or some mixed action, influences the outflow tract's fluid conductivity. Phosphotyrosine phosphatases, which have complex effects on tyrosine phosphorylation, are important regulators of actin and cell adhesion interactions (Geiger et al., 1995; Romer et al., 1992; Soulsby and Bennett, 2009). In cell culture, vanadate increases phosphotyrosine levels at adhesion sites and alters the actin cytoskeleton (Avalon and Geiger, 1997; Volberg et al,

1991). Separate evidence shows that ouabain, a Na⁺/K⁺-ATPase inhibitor, changes actin and focal adhesion organization in cultured TM cells and increases outflow facility in organ culture (Dismuke et al., 2009). Tyrosine phosphorylation may itself have a role in Na⁺/K⁺-ATPase regulation; for example, protein tyrosine phosphatase-1B increases Na⁺/K⁺-ATPase activity in lens fibers (Bozulic et al., 2004). While our study was not designed to tease out the complex roles of specific phosphorylation changes in regulating aqueous humor outflow resistance or the exact site of action of vanadate, these factors will be worth exploring in future studies.

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FIGURE 1.

IOP after topical treatment of monkey eyes in vivo with 1% Na_3VO_4 2×20 μL drops ~1 min apart. Treated and control eyes on day 1 (**A**) and day 4 (**B**) after twice daily treatment; **C**: Change in IOP in treated and control eyes on day 4 corrected for the BL prior to the first treatment; and **D**: IOP difference on day 4 between treated and control eyes corrected for BL prior to the first treatment. Data are mean $\pm$ s.e.m. IOP at each time-point is for n monkeys, each animal contributing one treated and one vehicle-control eye. Dotted lines indicate initial pre-treatment BL IOP on day 1 (**A**) or day 4 (**B**). BL = baseline. Significantly different from 0.0: $\emptyset$ $p \leq 0.05$; † $p \leq 0.005$; ‡ $p \leq 0.001$.

FIGURE 2.

Immunofluorescence of the actin cytoskeleton (F-actin) of HTM cells in untreated control cells (**A**) and cells treated with 1 mM Na_3VO_4 for 24h (**B**); vinculin-containing cell adhesions of untreated control cells (**C**) and cells treated with 1 mM Na_3VO_4 for 24h (**D**). Merged higher magnification (1000 $\times$) images are of the actin cytoskeleton (green), vinculin-containing adhesions (orange) and nuclei (blue Hoechst stain) in control (**E**) and Na_3VO_4 -treated cells (**F**). After exchanging Na_3VO_4 -containing medium with Na_3VO_4 -free medium, the disrupted actin cytoskeleton (**G**), vinculin-containing adhesions (**H**) and cell monolayers reformed over time.

TABLE 1

Total outflow facility (OF) ($\mu\text{l}/\text{min}/\text{mmHg}$) at baseline and after anterior chamber exchange with 100 μM or 1 mM sodium orthovanadate (Na_3VO_4) or vehicle.

	Outflow facility ($\mu\text{l}/\text{min}/\text{mmHg}$)		
	Treated (Na_3VO_4)	Control (vehicle)	Treated/Control
Baseline (n = 5)	0.46 ± 0.09	0.48 ± 0.10	1.01 ± 0.11
Baseline (n = 12)	0.48 ± 0.06	0.44 ± 0.06	1.15 ± 0.08
100 μM Na_3VO_4 (n = 5)	0.60 ± 0.15	0.50 ± 0.12	1.29 ± 0.22
1 mM Na_3VO_4 (n = 12)	0.76 ± 0.12	0.57 ± 0.10	$1.44 \pm 0.10^\ddagger$
100 μM Na_3VO_4 /Baseline	1.24 ± 0.12	0.99 ± 0.08	1.26 ± 0.10
1 mM Na_3VO_4 /Baseline	$1.53 \pm 0.17^\S$	1.20 ± 0.12	$1.29 \pm 0.09^\S$

Data are shown as mean $\pm$ s.e.m. for n monkeys, each contributing one drug-treated and one vehicle-treated eye. Ratios are unitless.

§ $p \leq 0.01$;

‡ $p \leq 0.005$.