Multimodal Fundus Imaging of Sodium Iodate-Treated Mice Informs RPE Susceptibility and Origins of Increased Fundus Autofluorescence

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Submitted: January 25, 2017
Accepted: March 15, 2017
Citation: Zhao J, Kim HJ, Sparrow JR. Multimodal fundus imaging of sodium iodate-treated mice informs RPE susceptibility and origins of increased fundus autofluorescence. Invest Ophthalmo Vis Sci. 2017;58:2152–2159. DOI:10.1167/iovs.17-21557

When delivered systemically, sodium iodate (NaIO₃), a strong oxidant, is known to primarily target RPE cells with effects on photoreceptor cells occurring secondarily.¹⁻⁶ In recent years, NaIO₃ administration in rats and mice has been used to model the atrophic lesions that are a feature of AMD.¹⁻⁶ The choriocapillaris underlying RPE also undergoes atrophy. Central retina is damaged preferentially.¹¹⁻¹² The structural changes resulting from NaIO₃ retinal damage have been extensively reported. Following a single injection of NaIO₃, degeneration begins with the RPE cell followed by loss of photoreceptor cell nuclei in subjacent outer nuclear layer (ONL).¹¹ The choriocapillaris underlying RPE also undergoes atrophy. Central retina is damaged preferentially.¹¹⁻¹² The changes can be monitored by ERG, by measuring visual acuity (optomotor reflex) by light microscopy, TUNEL assay, and by measuring visual acuity (optomotor reflex) and visual function.

Several mechanisms have been associated with NaIO₃-mediated damage.¹²⁻¹⁹ with the differences in pathways followed being dose-associated.¹³⁻¹⁹ For instance, NaIO₃-stress activates the AKT/mammalian target of rapamycin (AKT/mTOR) signaling pathway in RPE, whereas rapamycin delivered to inhibit mTOR attenuates NaIO₃-associated retinal degeneration.²⁰ Observations made after delivering a low dose of NaIO₃ (20 mg/kg) to mice deficient in zB crystallin indicate that this small heat shock protein may provide protection against oxidative stress by upregulating AKT phosphorylation and peroxisome proliferator-activator receptor-γ expression.³¹ NaIO₃ is known to directly oxidize thiol (-SH) groups resulting in an increase in the number of disulphide (S-S) bonds.²¹ Moreover, cotreatment with cysteine or glutathione reduces the damage to RPE caused by NaIO₃.²² When oxidative stress is induced with NaIO₃, an age-related increase in superoxide anion and malondialdehyde is observed.¹³ It is also reported that murine and human RPE cells cultured in the presence of NaIO₃ are induced to generate reactive oxygen species.³³ Additionally, NaIO₃ is said to denature protein.²⁵

Interactions between NaIO₃ and light also have been shown with the effects of NaIO₃ being greater in mice exposed to bright light.²⁴ The impact is also greater in albino mice and rats as opposed to pigmented rodents.¹⁵ When the effects of NaIO₃ were compared in young (2 month) versus older (15 month) mice, age-associated effects were observed.¹³ NaIO₃ is an oxidizing agent due to its ability to extract electrons, but why the RPE is particularly susceptible to NaIO₃-induced damage is not clear. Besides the metabolic sources of oxidative stress, RPE cells are subject to photooxidative damage.
originating in the bisretinoid fluorophores that form in photoreceptor cells and accumulate with age as the lipofuscin of RPE cells. Short-wavelength fundus autofluorescence (SW-AF) originates primarily from these fluorophores in RPE. In some retinal disorders, such as acute macular neuroretinopathy and fundus flecks in Stargardt disease 1 (STGD1), we have noted localized elevations in SW-AF and proposed that this aberrant SW-AF may be a sign that photoreceptor cells are incapacitated and thus unable to expend the energy necessary to reduce visual cycle–derived retinaldehyde to nontoxic retinol. Thus, our objective here has been 2-fold. First, we tested for whether bisretinoid formation is increased when photoreceptor degeneration is induced, in this case degeneration secondary to NaIO3-associated RPE loss. We addressed this question by examining for evidence of enhanced SW-AF as measured by an established protocol (quantitative fundus autofluorescence [qAF]). The second question we addressed was whether RPE cells are susceptible to NaIO3 because of preexisting stress imposed by the photooxidative processes initiated by RPE bisretinoids. To this end, we studied the effects of NaIO3 administration in mice having elevated levels of bisretinoid lipofuscin (Abca4−/− mice), wild-type levels of bisretinoid lipofuscin, and mice (Rpe65+/−) that do not generate the visual cycle adducts constituting bisretinoid lipofuscin.

**METHODS**

**Animals**

Agouti 129S-Abca4tm1Ght/J mice and their control 129SvImJ mice, as well as pigmented Rpe65+/− mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and bred in-house. All mice were housed under 12-hour on-off cyclic lighting with in-cage illuminance of approximately 40 lux. A sterile solution of NaIO3 (Sigma-Aldrich Corp., St. Louis, MO, USA) was freshly prepared in PBS. NaIO3 was administered as a single intraperitoneal injection (30 mg/kg body weight). Animal protocols were approved by the Institutional Animal Care and Use Committee of Columbia University and complied with guidelines set forth by the ARVO Animal Statement for the Use of Animals in Ophthalmic and Vision Research.

**Fundus Imaging**

Mice were anesthetized, pupils were dilated, the cornea was lubricated, and mice were positioned as previously described. Fundus AF images (55° widefield lens; 0.98-mm detection pupil) at 488 nm and 790 nm excitation were obtained with a confocal scanning laser ophthalmoscope (Spectralis HRA; Heidelberg Engineering, Heidelberg, Germany) with laser power set at approximately 280 μW and sensitivity at 100 and 105, respectively, after visual pigment was bleached for 20 seconds. Nine successive frames were acquired at 488 nm excitation with the high-speed mode, and frames were saved in non-normalized mode. A mean of 100 frames at 790 nm excitation with high-speed automatic real-time mode, and resized with Photoshop CS4 was used for analysis. Quantitative Ultra-Performance Liquid Chromatography (UPLC)

Analysis was performed on a Waters Acquity UPLC system (Waters Corp, Milford, MA, USA) coupled on-line with a photodiode array detector. For elution, a Waters XBridge C18 reversed-phase column (2.5 μm, 3 × 50 mm) was used with a mobile phase of acetonitrile/methanol (1:1) in water with 0.1% formic acid (0–1 minute, 70% acetonitrile/methanol [1:1] in water; 1–27 minutes, 98% acetonitrile/methanol [1:1] in water; 27–30 minutes, acetonitrile/methanol [1:1]; flow rate of 0.5 mL/min) and injection volume of 5 μL. A2E peak area was integrated from UPLC chromatograms by using Waters Empower software.
In Vitro Experiments

In an in vitro photooxidation assay, synthesized A2E (50 μM)34 in 1% dimethyl sulfoxide (DMSO) in PBS without and with NaIO3 (5–500 μM, as indicated) was irradiated (430 ± 30 nm, 60s), and quantified. In other experiments, A2E (50 μM) in PBS with 1% DMSO was incubated (room temperature in the dark) with NaIO3 (100, 200 μM, as indicated) for 4 hours; controls were incubated without NaIO3. To test for direct effects of NaIO3 on cultured RPE, ARPE-19 cells (American Type Culture Collection, Manassas, VA, USA) deficient in endogenous lipofuscin34 were grown to confluence in 96-well plates as described.35 The cells accumulated A2E by delivery in culture media (3 μM) over 14 days. After culturing for an additional 5 days in media without A2E, the cells were incubated with NaIO3 (25, 250 μM and 2.5 mM) for 24 hours and then exposed to 430-nm light (±30 nm, time) and after 18 hours, MTT (4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide) assay (Roche Diagnostics, Indianapolis, IN, USA) was performed to evaluate cell viability.

Statistical Analysis

Statistical analysis was performed by using GraphPad Prism, version 6 (GraphPad Software, Inc., La Jolla, CA, USA); \( P < 0.05 \) was considered significant.

RESULTS

The effects of NaIO3 administration are well known to depend on the dose and route of administration.3,5,36 In initial experiments, we injected mice intraperitoneally with NaIO3 at concentrations of 30 and 60 mg/kg to determine an appropriate dose of intraperitoneal NaIO3 that would be delivered by single injection. We selected the 30 mg/kg dose for all further experiments because it produced moderate degeneration that could be detected 7 days after injection in all injected mice.

Fundus Imaging

To evaluate the effects of a moderate dose of NaIO3 on retina, we acquired fundus images of the Abca4−/−, wild-type, and Rpe65rd12−/− mice (age 6 months) using the NIR-R (820 nm), SW-AF (488 nm), and NIR-AF (790 nm) modalities (Fig. 1A). Fundus AF generated by 488-nm excitation originates predominantly from the bisretinoids that accumulate in RPE as lipofuscin.37 AF (488 nm), and NIR-AF (790 nm) modalities (Fig. 1A). Fundus Imaging

Disuniformity in the NIR-AF images was suggestive of changes in the melanin-containing RPE (Fig. 1A). Good correspondence between SW-AF and NIR-AF patterns was observed (Fig. 1A: 488 and 790 nm). Thus, darkened foci in SW-AF images colocalized with areas of markedly decreased AF in NIR-AF images acquired from Abca4−/− mice 7 days after NaIO3 injection. The bright areas of motting in the NIR-AF images also corresponded to brightness in the SW-AF. The affected versus nonaffected areas of retina were easier to delineate in the NIR-AF images due to greater contrast. These changes in the NIR-AF images were more marked in Abca4−/− mice than in wild-type and Rpe65rd12−/− mice. Seven days after NaIO3 injection in Rpe65rd12−/− mice, areas of aberrant increased and decreased AF signal were visible in the NIR-AF images, probably indicating loss and clumping of RPE cells, respectively.

There were no obvious contrast changes in NIR-R (820 nm) images (Fig. 1A). Given that the signal arises from deeper layers in the NIR-R images, choroidal vessels were occasionally visible.

Quantitative Fundus AF

SW-AF intensities were measured as qAF before NaIO3 (30 mg/kg) injection, 3 days after the injection, and 7 days after the injections (Fig. 1B). As reported previously,35 qAF intensities were more pronounced in Abca4−/− mice than in Abca4 wild-type mice at the same age. The more robust qAF
intensity in Abca4−/− mice reflects the well-known accelerated accumulation of bisretinoid lipofuscin as a result of ABCA4 deficiency. In Abca4−/− mice (age 5–6 months) treated with NaIO3 at 30 mg/kg, SW-AF intensity was increased 3 and 7 days after injection (P < 0.05; 1-way ANOVA and Tukey’s multiple comparison test). Although in wild-type mice there was a small increase in qAF between 3 and 7 days after injection, this difference was not statistically significant (P > 0.05) (Fig. 1B). As expected, minimal SW-AF signal was recorded in Rpe65rd12 mice due to a paucity of bisretinoid lipofuscin formation.

In all three mouse lines (Abca4−/−, wild-type, and Rpe65rd12) NIR-AF measured with 790-nm excitation, was reduced 3 days after NaIO3 injection (Fig. 1C). In agouti Abca4−/− and Rpe65rd12 mice, NIR-AF levels 7 days after injection were at the same level as observed 3 days’ postinjection, whereas in wild-type mice, NIR-AF intensity underwent an increase between 3 and 7 days postinjection.

Flat-Mounts

Flat-mounts consisting of RPE, choroid, and sclera were stained with antibody to ZO-1, a protein component of tight junctions, together with nuclear staining by DAPI. In flat-mounts from uninjected eyes of wild-type mice (age 6 months, 2 mice each), the RPE monolayer presented as a regularly arranged cobblestone-like arrangement of hexagonal-shaped cells of similar size (Fig. 2). The DAPI-stained nuclei were relatively uniformly distributed. The posterior eyecups of Abca4−/− mice also exhibited positive ZO-1 staining of polygonal-shaped RPE, although in this case the staining was punctate. In addition, the SW-AF of lipofuscin was visible. In eyes of the NaIO3-treated Abca4−/− and wild-type mice, the regular cellular mosaic was lost, patchy loss of cells was readily visible, and a granular AF was notable. Large patches of the RPE monolayer were denuded of cells in retinas from NaIO3-treated mice. The regularity of ZO-1 staining was largely disrupted in the Abca4−/− and wild-type NaIO3-injected mice after 7 days, and clumps of densely packed nuclei were visible. RPE cells in the periphery appeared elongated. Unexplained nonspecific nuclear staining was observed in the ZO-1-labeled preparations. Because similar staining has been noted in the literature, nonspecific antibody binding is the likely cause.

Light Microscopic Imaging

The effects of a single systemic injection of NaIO3 were also visible by light microscopy as progressive RPE and photoreceptor cell degeneration in all injected mice (Fig. 3). Although in hematoxylin and eosin–stained sections of control retina (noninjected) the RPE monolayer consisted of a continuous layer of contiguous cells, in NaIO3-treated retina, the RPE was severely altered as revealed by thinning of the RPE monolayer and gaps in the regular distribution of hematoxylin and eosin-stained RPE nuclei. These features were indicative of RPE loss. The changes in melanin pigment distribution indicated that in some locations, RPE appeared to have migrated anteriorly as previously reported, whereas at other sites, aggregations of RPE cells were visible.

Progressive disorganization of photoreceptor outer and inner segments was indicated by loss of alignment of outer and inner segments. Rosette-like rearrangements of photoreceptor cells in outer retina were also observed as reported previously. In areas of absent RPE, ONL appears to have collapsed such that photoreceptor cell nuclei came in contact with Bruch’s membrane. Thinning of the ONL in NaIO3-treated agouti Abca4−/− mice was more pronounced than in treated wild-type mice.
Measurement of ONL Thickness

To further assess the effect of NaIO₃ on outer neural retina, we evaluated photoreceptor cell viability in Abca4⁻/⁻, wild-type, and Rpe65rd12 mice by measuring ONL thickness. ONL thickness in 6-month-old agouti Abca4⁻/⁻ mice treated with NaIO₃ was reduced versus the nontreated Abca4⁻/⁻ mice and was also lower than in treated wild-type and Rpe65rd12 mice of the same age (Fig. 4A).

For comparative purposes, we calculated ONL area from the ONH to a distance of 2 mm in a superior and inferior direction (Fig. 4B). ONL area in NaIO₃-treated Abca4⁻/⁻ mice was significantly lower than in untreated Abca4⁻/⁻ mice (P < 0.05). Of particular interest, the decline in ONL area in NaIO₃-treated Abca4⁻/⁻ mice was also significantly lower than in NaIO₃-treated wild-type and Rpe65rd12 mice (P < 0.05, 1-way analysis of variance and Tukey's multiple comparison test). It is important to note that NaIO₃-induced ONL thinning in 5- to 6-month-old agouti Abca4⁻/⁻ mice was accompanied by increased SW-AF (488 nm) (Fig. 1B).

In Vitro Studies

For additional mechanistic studies, we examined NaIO₃ activity in vitro (Fig. 5). Delivery of NaIO₃ alone to ARPE-19 cells at a concentration of 25 and 250 µM did not cause the death of ARPE-19 cells, but at 2.5 mM approximately 40% loss of viability was observed. On the other hand, intracellular A2E combined with NaIO₃ (25 and 250 µM and 2.5 mM in the absence of 488-nm light exposure), was not associated with a further reduction in cell viability (Fig. 5A).

In ARPE-19 cells stressed by A2E accumulation and exposure to 430-nm light, viability was reduced by approximately 38% (Fig. 5A, solid blue bar). This finding is consistent with published results. Moreover, this loss of cell viability was exacerbated when the oxidizing environment of the cell was potentiated by a combination of NaIO₃ together with the photooxidation of intracellular A2E. Thus, when NaIO₃ was delivered to A2E-containing ARPE-19 and the cells were exposed to 430-nm light to induce A2E photooxidation, cell viability was reduced by 57% (250 µM NaIO₃) and 64% (2.5 µM NaIO₃) (P < 0.0001, 1-way ANOVA and Tukey's multiple comparison test). Conversely, when the loss of A2E due to photooxidation was measured chromatographically, we found no evidence that NaIO₃ could potentiate the photooxidation of A2E in a cell-free environment, even at the high concentration of 500 µM (Fig. 5B), nor did NaIO₃ (100 and 200 µM) alone directly oxidize A2E in a cell-free environment in the absence of light exposure.

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Fundus Imaging With Systemic Sodium Iodate in Mice

**Figure 5.** In vitro assays of NaIO3 (SI). (A) SI at indicated concentrations was incubated with ARPE-19 cells that had not or had accumulated A2E (bars outlined in orange) and were exposed or not exposed to 430-nm (≥30 nm) (blue bars) light for 20 minutes. Viability was determined by MTT absorbance (570 nm). Mean ± SEM of six replicates. P values determined by 1-way ANOVA and Tukey’s multiple comparison test. (B) In a cell-free assay, SI at indicated concentrations was combined with A2E (50 μM; bars outlined in orange) and was or was not (control) exposed to 430-nm light (60 seconds; blue bars). A2E photooxidation was assayed by UPLC measurement of A2E loss. SI does not potentiate the photooxidation of A2E. Mean ± SEM of two replicates. P > 0.05, 1-way ANOVA, and Tukey’s multiple comparison test. (C) In a cell-free assay, incubation of A2E (50 μM; bars outlined in orange) with SI at indicated concentrations for 4 hours does not result in oxidative loss of A2E measured by UPLC. Mean ± SEM of two replicates. P > 0.05, 1-way ANOVA, and Tukey’s multiple comparison test.

Effects of NaIO3 that are evident in the SW-AF (488 nm) images of NaIO3-treated Abca4−/− mice as compared with Abca4+/+ mice are not just attributable to the greater SW-AF signal in the mutant mice because even in NIR-AF images presenting signal that primarily originates from melanin, the disruptive effects of NaIO3 were more apparent in the NIR-AF images acquired from Abca4−/− mice. This abrupt rise in qAF cannot be attributed to the normal age-related increase in SW-AF in mice. Instead, because this increase coincided with ONL thinning, which is indicative of declining photoreceptor cell function and survival, elevated fundus AF due to augmented bisretinoid formation in stressed photoreceptor cells could explain these findings. These circumstances are significant. Elevated formation of these toxic photoreactive molecules could further accelerate photoreceptor degeneration.

**DISCUSSION**

As with genetic ablation of RPE, results in secondary degeneration of photoreceptor cells, NaIO3-induced retinal degeneration begins with RPE cell dysfunction and loss. Indeed, NaIO3-induced retinal degeneration is considered by some investigators to replicate the stage of degeneration observed in atrophic AMD. In an effort to understand the primary vulnerability of RPE to the oxidative stress imposed by systemic delivery of NaIO3, we tested the premise that the underlying stress imposed by the photoactivity of RPE bisretinoids could play a role. Thus, we used mice having elevated levels of bisretinoid lipofuscin (Abca4−/− mice), mice having wild-type levels, and mice deficient in bisretinoid lipofuscin (Rpe65−/−/− mice), and observed that the degenerative changes were most marked in Abca4−/− mice. Exploration of mechanisms in cultured RPE revealed that the combination of NaIO3 together with A2E and 430-nm light exposure to create photooxidizing conditions produced an additive effect, whereby levels of cell death were greater than A2E/430 nm alone or NaIO3 alone. Previous reports that light exposure and absence of ocular melanin (albinism) promote the effects of NaIO3 injection are consistent with our results indicating that underlying photooxidative stress from bisretinoid lipofuscin increases susceptibility of RPE to NaIO3-associated atrophy. It seems that the additive effects of bisretinoid and NaIO3 are manifest at milder concentrations of NaIO3, whereas at high concentrations, NaIO3 alone can impose damage (Fig. 5A). The combined effects of bisretinoid phototoxic stress and NaIO3 likely compromise cellular oxidant defense. For instance, both sources of stress can reduce intracellular glutathione levels and the damage mediated by both NaIO3 and bisretinoid photooxidative damage can be ameliorated by chelation of labile iron. The pronounced ONL thinning that we observed is consistent with the view that RPE cell dysfunction leading to photoreceptor cell degeneration can precede overt RPE cell loss.

The fluorescent bisretinoids of RPE lipofuscin that are the source of SW-AF fundus AF undergo nonenzymatic formation in photoreceptor cell outer segments. Due to daily shedding of outer segment membrane, the fluorophores then undergo phagocytic transfer to RPE where they accumulate. Because bisretinoid formation precedes RPE phagocytosis, the latter process is unlikely to be a determinant of the rate of RPE lipofuscin formation. We previously proposed that SW-AF fundus AF intensity does not just signal the status of RPE but can under some circumstances be indicative of the health of photoreceptor cells. As a test of our hypothesis, here we analyzed SW-AF intensities in mice undergoing NaIO3-induced photoreceptor degeneration. We observed a 36% increase in SW-AF intensity within 3 days after injection of NaIO3 in agouti Abca4−/− mice. This abrupt rise in qAF cannot be attributed to the normal age-related increase in SW-AF in mice. Instead, because this increase coincided with ONL thinning, which is indicative of declining photoreceptor cell function and survival, elevated fundus AF due to augmented bisretinoid formation in stressed photoreceptor cells could explain these findings. These circumstances are significant. Elevated formation of these toxic photoreactive molecules could further accelerate photoreceptor degeneration.

The effects of NaIO3 that are evident in the SW-AF (488 nm) images of NaIO3-treated Abca4−/− mice as compared with Abca4+/+ mice are not just attributable to the greater SW-AF signal in the mutant mice because even in NIR-AF images presenting signal that primarily originates from melanin, the disruptive effects of NaIO3 were more apparent in the NIR-AF images acquired from Abca4−/− mice. Thus, multimodal imaging gives emphasis to the more pronounced effects of NaIO3 in mice having impaired oxidant defense (receptor-interacting protein kinase-1) has also been shown to rescue RPE from death. From numerous studies over the years, reports have emerged regarding the impact of NaIO3 on retina. The work presented here has further advanced our understanding of this model. Using both in vitro and mouse models, interactions between the oxidative effects of NaIO3 on RPE and photooxidative stress imposed by lipofuscin bisretinoid also have been revealed. We conclude that photooxidative stress inflicted by RPE bisretinoid lipofuscin contributes to the disposition of RPE toward NaIO3 toxicity. We also suggest that aberrant SW-AF under some conditions may be a sign of impaired photoreceptor cells. These results may impact the interpretations of SW-AF in some retinal disorders.
Acknowledgments
Supported by grants from the National Eye Institute (EY12951 and P40EY019007) and Foundation Fighting Blindness, and a grant from Research to Prevent Blindness to the Department of Ophthalmology, Columbia University.
Disclosure: J. Zhao, None; H.J. Kim, None; J.R. Sparrow, None

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