Mechanisms of adaptation of the retina to oxidant stress

Robert E. Anderson, Masaki Tanito,
Sachiko Kaidzu & Akihiro Ohira

Abstract

One of the dilemmas in research on omega-6 and omega-3 poly-unsaturated fatty acid (PUFA) metabolism is how these fatty acids, which are so susceptible to non-enzymatic oxidation, can be neuroprotective in an oxidant stress environment. To address this issue in the retina, we designed a series of experiments to test the hypothesis that the products of PUFA oxidation up-regulate the expression of endogenous neuroprotective pathways. 4-Hydroxynonenal (4-HNE) and 4-hydroxyhexenal (4-HHE) were produced during light stress and decorated retinal proteins. When provided in cell culture in sub-lethal amounts, 4-HNE protected the cells from subsequent challenge with hydrogen peroxide. The transcription factor Nrf2 was found to be up-regulated in the cells incubated in 4-HNE, and was responsible for increased expression of three proteins, thioredoxin (Trx), thioredoxin reductase (TrxR), and heme oxygenase-1 (HO-1).

ANDERSON, R. E., M. TANITO, S. KAIDZU, and A. OHIRA. 2007. Mechanisms of adaptation of the retina to oxidant stress. *Biol. Skr. Dan. Vid. Selsk.* **56**: 33-41. ISSN 0366-3612 • ISBN 978-87-7304-327-1

Robert E. Anderson, Departments of Ophthalmology and Cell Biology, University of Oklahoma Health Sciences Center, Dean McGee Eye Institute, Oklahoma City, OK USA 73104 • Robert-Anderson@ouhsc.edu

Masaki Tanito, Sachiko Kaidzu, and Akihiro Ohira, Department of Ophthalmology, Shimane University Faculty of Medicine, Enya 89-1, Izumo, Shimane, 693-8501, Japan

Key words: retinal light adaptation, 4-hydroxynonenal (4-HNE); 4-hydroxyhexenal (4-HHE), nuclear-factor-E2-related factor 2 (Nrf2), lipid peroxidation, thioredoxin, thioredoxin reductase, heme oxygenase-1 (HO-1)

Introduction

The photosensitive rod outer segment (ROS) membranes of the retina contain the highest level of DHA of any mammalian membrane (Fliesler and Anderson, 1983). Over 30 years ago, we established a role for DHA in retinal function by showing that dietary restriction of omega-3 PUFA in rats led to a reduced a- and b-wave of their electroretinogram (Benolken *et al.*, 1973; Wheeler *et al.*, 1975). Other laboratories made the same observation in rats (Watanabe *et al.*, 1987; Bourre *et al.*, 1989) and guinea pigs (Weisinger *et al.*, 1996).

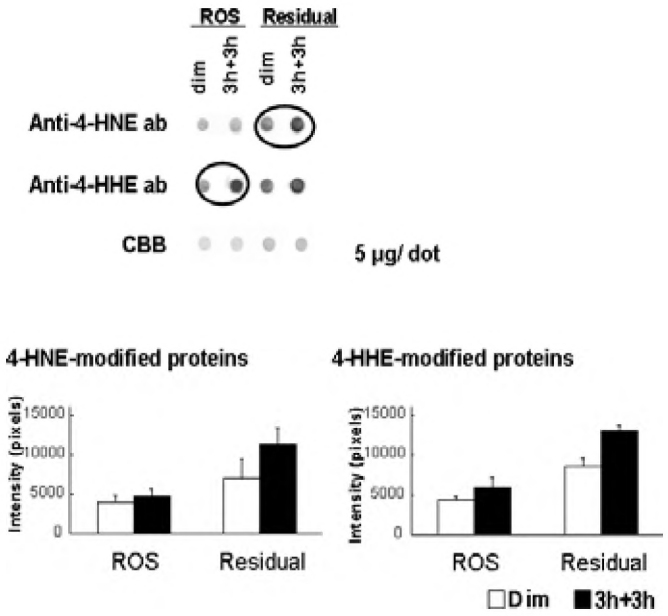

Fig. 1. Western dot blot for 4-HNE- and 4-HHE-protein modifications in ROS and residual retinal fractions. The eyes were enucleated before (dim, 5 lux) and after 3h of light exposure at 5,000 lux followed by 3h of dim light. The circled areas illustrate dim/bright differences. (Upper panel)-Representative western dot blots of ROS and residual retinal fractions. CBB, Coomassie Brilliant Blue. (Lower panel)- Densitometric analysis of dots. [Reprinted from Tanito *et al.* (2005a) with permission from Invest. Ophthalmol. Vis. Sci.]

Studies in monkeys (Neuringer *et al.,* 1984) and human infants (Carlson *et al.,* 1993; Uauy *et al.,* 1990; Birch *et al.,* 1992; 1998) demonstrated the importance of dietary sources of omega-3 fatty acids in the development of the visual system. Thus, development and optimal electrical function of the retina depend on an adequate supply of DHA in the developing fetus and in the diet thereafter.

DHA has also been found to have a neuroprotective function in retinal neurons. Supplementation of cultured retinal neurons with DHA protects the cells from oxidant stress-induced apoptosis (Rotstein *et al.,* 1997; 2003). A molecular explanation for this effect was recently demonstrated in a seminal paper by Mukherjee *et al.* (2004) that described the stress-induced production of a bioactive molecule from DHA, which was named neuroprotectin D1 (see review by Bazan (2007) and Dr. Bazan's chapter in this volume).

The retina exists in a paradoxical state. On the one hand, it is challenged daily with light and a high oxygen flux, which work in concert to produce reactive oxygen species that can destroy PUFA and lead to cell death. On the other hand, high levels of DHA are necessary for development and optimal function of the retina. To survive, the retina has established elaborate defense mechanisms that can protect it from oxidant stress-induced cell death.

Role of oxidant stress in retinal degeneration

Previous studies have suggested that oxidant stress plays a major role in retinal degenerations, including age-related macular degeneration (Winkler *et al.,* 1999; Beatty *et al.,* 2000) and diabetic retinopathy (van Reyk *et al.,* 2003; Kanwar *et al.,* 2007). We

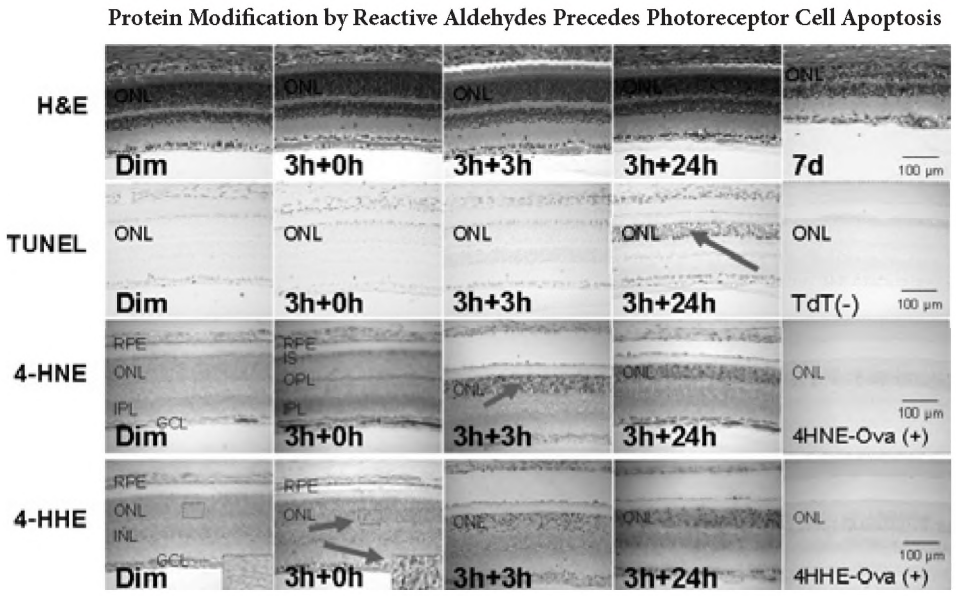

Fig. 2. H&E, TUNEL, and immunohistochemical staining for 4-HNE- and 4-HHE-protein modifications. Eyes were enucleated before (dim, 5 lux) and at 3h+0h (time in light stress followed by time in dim light), 3h+3h, 3h+24h, and 3h+7d of light exposure at 5,000 lux. Representative images at 1 mm superior from the optic nerve head are shown (5 rats analyzed in each group). (First panel)- H&E staining. Severe loss of ONL is observable at 3h+7d. (Second panel)- TUNEL staining. Nuclear staining was observed in ONL at 3h+24h (red arrow). TdT(-), no TdT enzyme (control) on the same section of 3h+24h. (Third panel)- Immunohistochemistry for 4-HNE-modified proteins. Nuclear staining was observed in the ONL at 3h+3h and later (blue arrow). The 4-HNE-OVA(+) blocking experiment was done on a 3h+24h section. (Lower panel)- Immunohistochemistry for 4-HHE-modified proteins. Nuclear/perinuclear staining was observed in the ONL (inset) at 3h+0h (blue arrows). Nuclear staining in the ONL became more dramatic at 3h+3h and 3h+24h. The 4-HHE-OVA(+) blocking experiment was done on a 3h+24h section. [Reprinted from Tanito *et al.* (2005a) with permission from Invest. Ophthalmol. Vis. Sci.]

study oxidant stress in the retina using an acute light damage model (Ranchon *et al.,* 2001). Albino rats born and raised in dim cyclic light will loose a significant number of their rod photoreceptors if placed in bright (usually 3,000-5,000 lux) for 3-6 hours. Under these conditions, the central superior region of the retina is most susceptible to damage. The role of lipid peroxidation in retinal degeneration was determined by studying the production and distribution of 4-hydroxynonenal (4-HNE) and 4-hydroxyhexenal (4-HHE), which are reactive aldehydes derived from the non-enzymatic oxidation of omega-6 and omega-3 PUFA, respectively. This review summarizes some of our recent studies (Tanito *et al.,* 2005a; Tanito *et al.,* 2006; Tanito *et al.,* 2007).

Albino rats were stressed in 5,000 lux light for 3 hours followed by 3 hours in dim light, after which retinas were removed, homogenized, and assessed by densitometric analysis of semi-quantitative western dot blots using 4-HNE and 4-HHE specific antibodies. Protein modifications by 4-HNE and 4-HHE increased in retinal tissues after exposure of rats to high intensity light (Fig. 1), showing that acute light stress leads to lipid peroxidation in the retina.

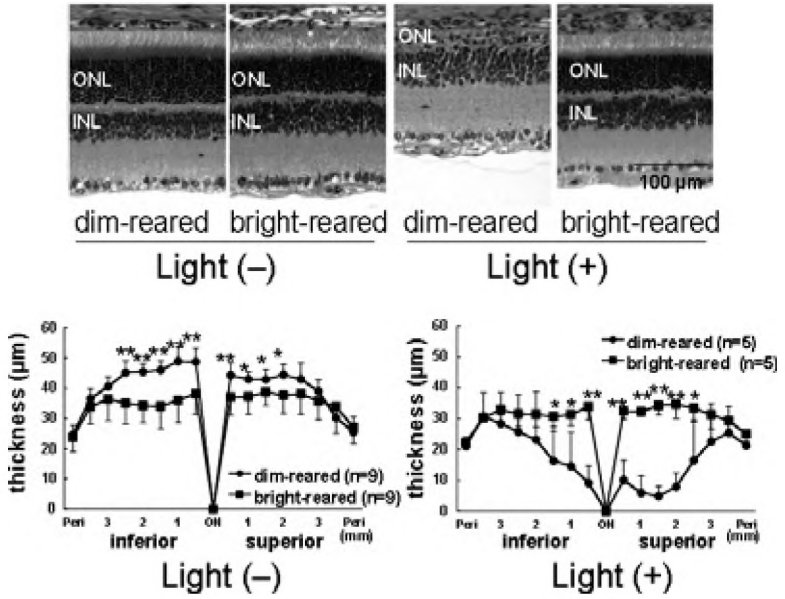

Fig. 3. Quantification of the outer nuclear layer thickness and area. (Upper panel)- Representative retinal sections of at 1-1.5 mm superior to the optic nerve head stained with H&E. ONL, outer nuclear layer; INL, inner nuclear layer. Note the severe loss of rod nuclei (ONL) in the dim-reared exposed retina. (Lower panels)- ONL thickness in dim light-reared and bright light-reared groups without (left panel) and after (right panel) damaging light exposure. The mean (±SD) thickness is shown. The * and ** indicate significant differences ($p<0.05$ and $p<0.01$, respectively) between the dim light-reared (5 lux) and the bright light-reared (400 lux) groups using an un-paired t-test. The number of animals analyzed is indicated in each graph. Significant differences are indicated by * and ** for $p<0.05$ and $p<0.01$, respectively, using a 1-way ANOVA followed by Scheffe's posthoc test. The number of animals analyzed is indicated in each graph. [Reprinted from Tanito *et al.* (2007) with permission from Elsevier]

To determine the retinal localization of 4-HNE and 4-HHE following bright light stress, albino rats were exposed to 5,000 lux light for 3h and, at various times thereafter, the levels and localizations of aldehyde-modified proteins in retinas was determined by immunohistochemistry using 4-HNE- and 4-HHE-specific antibodies (Fig. 2). Increases in 4-HNE- and 4-HHE-modified proteins were more prominent at 3h than at 24h following light exposure, and preceded rod photoreceptor cell apoptosis, determined by TUNEL staining. We conclude that intense light exposure increases 4-HNE- and 4-HHE-protein modifications in the retina, suggesting that free radical initiated, non-enzymatic reaction oxidation of omega-6 and omega-3 PUFA is involved in this process. These modifications are early events that precede photoreceptor cell apoptosis, consistent with our hypothesis that oxidant stress leads to photoreceptor cell death in our light stress model.

The dilemma

In light of the above-described studies, how then can PUFA be neuroprotective in the retina under oxidant stress conditions (Rotstein *et al.,* 1997; Rotstein *et al.,* 2003; Bazan, 2007)? We have previously shown that albino rats born and raised in dim (5 – 10 lux) cyclic light were more susceptible to light-induced apoptosis that albino rats born

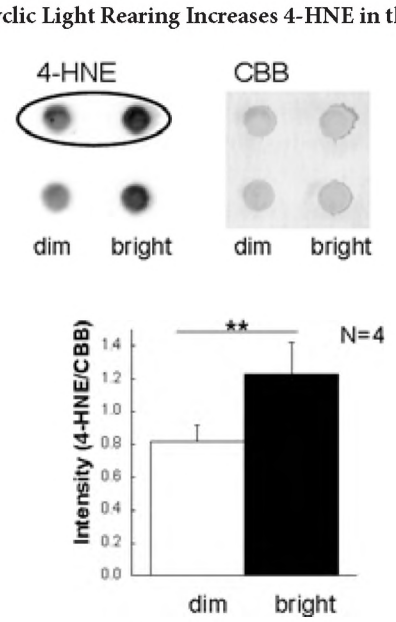

Fig. 4. Western dot blots and immunohistochemical localization of 4-HNE in the retina. (Upper panel)- Representative dot blots of protein modifications by 4-HNE for the dim light-reared (5 lux) and the bright light-reared (400 lux) groups. Note the difference between the circled dot-blots. CBB staining served as a loading control. (Lower panel)- Densitometric analysis of blots. The mean (±SD) densities standardized using CBB staining are shown for the dim light-reared and the bright light-reared groups (n=4 rats in each group). The ** indicates significant differences ($p<0.01$) between the two groups, using an un-paired t-test. [Reprinted from Tanito *et al.* (2007) with permission from Elsevier]

and raised in bright (300-800 lux) cyclic light (Penn *et al.,* 1987). Biochemical studies showed an up-regulation of antioxidants and the activity of glutathione-metabolizing enzymes (Penn and Anderson, 1987). In a later study, we continued and expanded these earlier investigations to address the molecular mechanisms involved in this phenomenon. Specifically, we tested the hypothesis that stress responses mediated by the Nrf2-antioxidant responsive element (ARE) pathway are involved in the initiation of retinal neuroprotection provided by bright cyclic light-rearing (Tanito *et al.,* 2007). Albino rats born and raised in dim (5 lux) or bright (400 lux) cyclic light were exposed to damaging light (3000 lux, 6 h). After exposure, the outer nuclear layer (ONL) thickness and area, and electroretinogram a- and b-wave amplitudes were significantly reduced in the dim light-reared rats compared to the bright light-reared rats, confirming a light adaptation neuroprotection phenomenon (Fig. 3).

Retinas from albino rats born and raised in dim or bright cyclic light were removed and analyzed for the presence of 4-HNE. Although protected from light stress-induced cell death, the retinas from rats raised in 400 lux had higher levels of 4-HNE (Fig. 4), indicating that the chronic light stress led to an increased non-enzymatic oxidation of omega-6 PUFA in the retina.

These results suggested that the production of

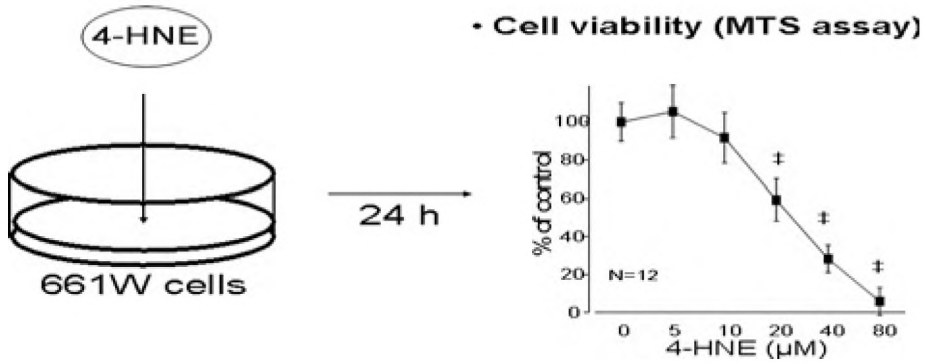

Fig. 5. Cell viability assay using 661W cells treated with various concentrations of 4-HNE for 24 h. The mean (±SD) for cell viability is shown (n=12 in each group). The ‡ indicates significant differences ($p<0.01$) in comparisons to cells that were not treated with 4-NHE using a 1-way ANOVA followed by Scheffe's posthoc test. [Reprinted from Tanito *et al.* (2007) with permission from Elsevier]

4-HNE in the bright cyclic light-raised rats may be involved in some paradoxical way in providing neuroprotection to the retinal photoreceptors when challenged by damaging levels of light. To test this hypothesis, we used cell culture of an immortalized line of cone photoreceptors (called 661W cells). There was a dose-dependant loss of cells treated with 4-HNE for 24 hr, indicating that this product of lipid peroxidation, shown above to accumulate during light stress, was cytotoxic to these cultured retinal neuronal cells (Fig. 5). However, at low doses, between 0 and 10 μM, 4-HNE was not cytotoxic.

We then tested the hypothesis that pretreatment with a sub-lethal dose of 4-HNE could protect against stress-induced cell damage. Cells were pretreated with 5 μM 4-HNE for 24 hours, after which they were exposed to different concentrations of H_2O_2 for an additional 24 hours (Fig. 6). Although cell death occurred at all peroxide concentrations, there was a significant protective effect of pretreatment with 4-HNE.

Tanito *et al.* (2005b) had previously shown that sulforaphane, an extract from broccoli, up-regulated retinal levels of Trx in rat retinas. Using our cell culture paradigm, we looked for the up-regulation of Trx, TrxR, and HO-1 following treatment with 5 μM 4-HNE for 24 hours (Fig. 7). All three were up-regulated, as was Nrf2, a nuclear transcription factor previously shown to be involved in Trx up-

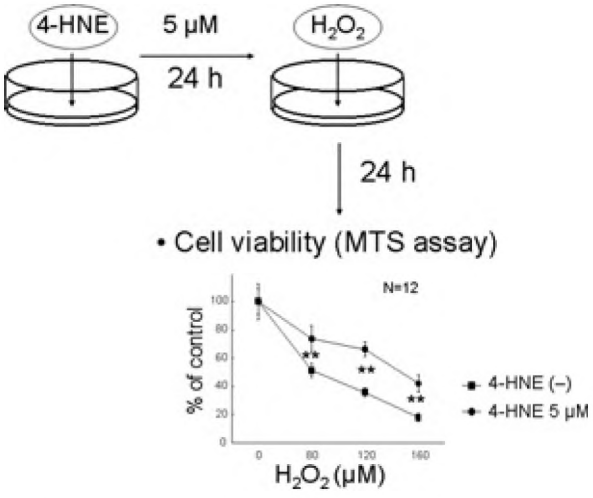

Fig. 6. Viability of 661W cells pretreated with 5 μM 4-HNE for 24 h followed by various concentrations of H_2O_2 for 24 h. The mean (±SD) for cell viability is shown (n=12 in each group). The ** indicates a significant difference ($p<0.01$) between the 4-HNE treated and untreated cells using an un-paired t-test. [Reprinted from Tanito *et al.* (2007) with permission from Elsevier]

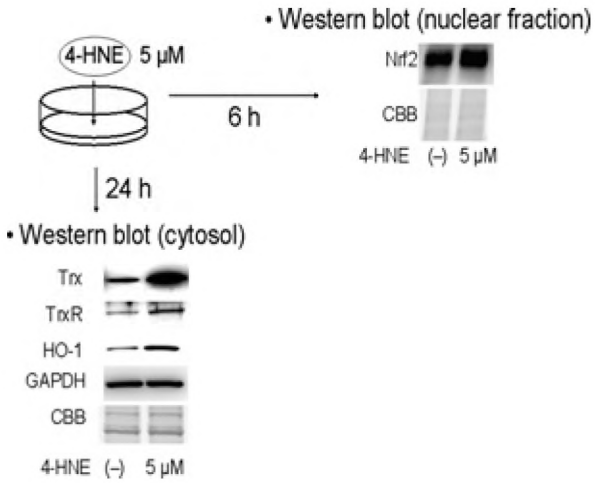

Fig. 7. Effect of 4-HNE on protein expression. (Upper right)- Western blot for Nrf2 in the nuclear fraction from 661W cells treated with or without 5 µM 4-HNE for 6 hours. CBB, Coomassie Brilliant Blue. (Lower left)- Western blots for Trx, TrxR, HO-1, and GAPDH using the cytosolic fraction from cells treated with or without 5 µM 4-HNE for 24 h. Note the increased immunoreactivity in the treated lane. (Lower right)- Densitometric analysis of blots from the western blots. The mean (±SD) densities standardized using GAPDH are shown (n=3 samples for each group). The * and ** indicate significant differences ($p<0.05$ and $p<0.01$, respectively) between the 4-HNE treated and untreated cells using an un-paired t-test. [Reprinted from Tanito *et al.* (2007) with permission from Elsevier]

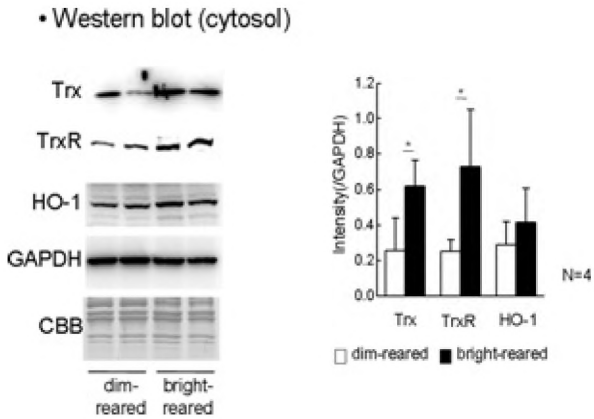

Fig. 8. Western blots for Trx, TrxR, HO-1, and GAPDH in the retinal cytosolic fraction from both the dim (5 lux) and bright (400) cyclic light-reared groups. CBB, Coomassie Brilliant Blue. (Right panel)- Densitometric analysis of Western blots. The mean (±SD) densities were standardized against GAPDH (n=4 rats for each group). The * indicates significant differences ($p<0.05$) between the two groups using an un-paired t-test. [Reprinted from Tanito *et al.* (2007) with permission from Elsevier]

regulation. Prevention of Nrf2 expression using siRNA technology also reduced the up-regulation of Trx and TrxR, but apparently not HO-1 (results not shown).

To determine if bright light rearing up-regulated Trx, TrxR, and HO-1 in animals, retinas from rats born and raised in 5 lux or 400 lux cyclic light were taken for semi-quantitative Western blot analysis (Fig. 8). Trx and TrxR were significantly increased in bright raised rat retinas. HO-1 was also higher, but the difference was not significant in the small sample size (n=4). The level of Nrf2 was also higher in nuclear extracts of retinas from rats born and raised in 400 lux cyclic light, compared to dim-reared (5 lux) controls (not shown).

Conclusions

Our studies show that products of the non-enzymatic oxidation of omega-6 and omega-3 PUFA can lead to the death of neural cells. However, we also demonstrate that sub-lethal doses of these products (in the reviewed studies we tested 4-HNE), which would occur in the initial phases of oxidant stress, can up-regulate endogenous neuroprotective pathways. We therefore suggest that in their day-to-day course of metabolic activity, cells challenged with an oxidant stress respond by up-regulating a host of endogenous neuroprotective mechanisms, which include Trx, TrxR, HO-1, GSH-peroxidase, GSH-reductase, GSH-S-transferase, vitamin E, and vitamin C, to name a few.

These, along with the enzymatic products of PUFA metabolism, such as neuroprotectin D1 (Bazan, 2007), serve to maintain the cell in a reduced state and protect against oxidant stress-induced apoptosis.

Acknowledgments

Mouse photoreceptor-derived 661W cells were kindly provided by Dr. Muayyad Al-Ubaidi (Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK). This study was supported by grants from the National Eye Institute (EY04149, EY00871, and EY12190); National Center for Research Resources (RR17703); Research to Prevent Blindness, Inc.; and the Foundation Fighting Blindness. Masaki Tanito was a recipient of a Research Fellowship from the Japan Society for the Promotion of Science (JSPS) for Young Scientists.

References

Bazan, N. G. Homeostatic regulation of photoreceptor cell integrity: significance of the potent mediator Neuroprotectin D1 biosynthesized from docosahexaenoic acid. The Proctor Lecture. *Invest. Ophthalmol. Vis. Sci.* **48**, 4866-4881 (2007).

Beatty, S., H. Kohl, M. Phil, D. Henson, and M. Boulton, The role of oxidative stress in the pathogenesis of age-related macular degeneration. *Surv. Ophthalmol.* **45**, 115-134 (2000).

Benolken, R. M., R. E. Anderson, and T. G. Wheeler, Membrane fatty acids associated with the electrical response in visual excitation. *Science* **182,** 1253-1254 (1973).

Birch, E. E., D. G.Birch, D. R. Hoffman, and R. Uauy, Dietary essential fatty acid supply and visual acuity development. *Invest. Ophthalmol. Vis. Sci.* **33**, 3242-3253 (1992).

Birch, E. E., D. R. Hoffman, R. Uauy, D. G. Birch, and C. Prestidge, Visual acuity and the essentiality of docosahexaenoic acid and arachidonic acid in the diet of term infants. *Pediatr. Res.* **44**, 201-209 (1998).

Bourre, J., M. Francois, A. Youyou, O. Doumont, M. Piciotti, G. Pascal, and G. Durand, The effects of dietary α-linolenic acid on the composition of nerve membranes, enzymatic activity, amplitude of electrophysiological parameters, resistance poisons and performance of learning tasks in rats. *J. Nutr.* **119**, 1880-1892 (1989).

Carlson, S. E., S. H. Werkman, P. G. Rhodes, and E. A. Tolley, Visual-acuity development in healthy preterm infants: Effect of marine oil supplementation. *Am. J. Clin. Nutr.* **58**, 35-42 (1993).

Fliesler, S. J. and R. E. Anderson, Chemistry and metabolism of lipids in the vertebrate retina. In *Progress in Lipid Research* (R. T. Holman. ed.) Pergamon Press, London, **22**, 79-131 (1983).

Kanwar, M., P. S. Chan, T. S. Kern, and R. A. Kowluru, Oxidative damage in the retinal mitochondria of diabetic mice: possible protection by superoxide dismutase. *Invest. Ophthalmol. Vis. Sci.* **48**, 3805-311 (2007).

Mukherjee, P. K., V. L. Marcheselli, C. N. Serhan, and N. G. Bazan, Neuroprotectin D1: a docosahexaenoic acid-derived docosatriene protects human retinal pigment epithelial cells from oxidative stress. *Proc. Natl. Acad. Sci. USA.* **101**, 8491-8496 (2004).

Neuringer, M., W. E. Connor, C. Van Patten, and L. Barstad, Dietary omega-3 fatty acid deficiency and visual loss in infant rhesus monkeys. *J. Clin. Invest.* **73**, 272-276 (1984).

Penn, J. S., M. I. Naash, and R. E. Anderson, Effect of light history on retinal antioxidants and light damage susceptibility in the rat. *Exp. Eye Res.* **44**, 779-788 (1987).

Penn, J. S. and R. E. Anderson, Effect of light history on rod outer segment membrane composition in the rat. *Exp. Eye Res.* **44**, 767-778 (1987).

Ranchon, I., S. Chen, K. Alvarez, and R. E. Anderson, Systemic administration of phenyl N-*tert*-butylnitrone (PBN) protects retinal rod photoreceptor cells from light damage. *Invest. Ophthalmol. Vis. Sci.* **42**, 1375-1379 (2001).

Rotstein, N. P., M. I. Aveldano, F. J. Barrantes, A. M. Roccamo, and L. E. Politi, Apoptosis of retinal photoreceptors during development in vitro: protective effect of docosahexaenoic acid. *J. Neurochem.* **69**, 504-513 (1997).

Rotstein, N. P., L. E. Politi, O. L. German, and R. Girotti, Protective effect of docosahexaenoic acid on oxidative stress-

induced apoptosis of retina photoreceptors. *Invest. Ophthalmol. Vis. Sci.* **44**, 2252-2259 (2003).

Tanito, M., M. H. Elliott, Y. Kotake, Y., and R. E. Anderson, Protein modifications by 4-hydroxynonenal and 4-hydroxyhexenal in light-exposed rat retina. *Invest. Ophthalmol. Vis. Sci.* **46**, 3859-3868 (2005a).

Tanito, M., H. Masutani, Y. C. Ki, M. Nishikawa, A. Ohira, and J. Yodoi, Sulforaphane induces thioredoxin through the antioxidant-responsive element and attenuates retinal light damage in mice. *Invest. Ophthalmol. Vis. Sci.* **46**, 979-987 (2005b).

Tanito, M., H. Haniu, M. H. Elliott, A. K. Singh, H. Matsumoto, and R. E. Anderson, Identification of 4-HNE-modified retinal proteins induced by photooxidative stress prior to retinal degeneration. *Free Radical Biology & Medicine* **41**, 1847-1859 (2006).

Tanito, M., M.-P. Agbaga, and R. E. Anderson, Upregulation of thioreodoxin system via Nrf-2-antioxidant responsive element pathway in bright cyclic light rearing-mediated retinal protection in vivo and in 4-hydroxynonenal-mediated adaptive response *in vitro. Free Radical Biology & Medicine* **42**, 1838-1850 (2007).

Uauy, R.D., D. G. Birch, E. E. Birch, J. E. Tyson, and D. R. Hoffman, Effect of dietary omega-3 fatty acids on retinal function of very-low-birth-weight neonates. *Pediatr. Res.* **28**, 485-492 (1990).

van Reyk, D. M., M. C. Gillies, and M. J. Davies, The retina: oxidative stress and diabetes. *Redox. Rep.* **8**, 187-192 (2003).

Watanabe, I., M. Kato. H. Aonuma, A. Hishimoto, Y. Naito, A. Moriuchi, and H. Okuyama, Effect of dietary alpha-linolenate/linoleate balance on the lipid composition and electroretinographic responses in rats. In *Advances in the Biosciences*, Vol. 62: *Research in Retinitis Pigmentosa* (E. Zrenner, H. Krastel, and H. H. Goebel, eds.) Pergamon Journals Ltd., Oxford, pp. 563-570 (1987).

Weisinger, H., A. Vingrys, and A. Sinclair, Effect of dietary n-3 deficiency on the electroretinogram in the guinea pig. *Ann. Nutr. Metab.* **40**, 91-98 (1996).

Wheeler, T. G., R. M. Benolken, and R. E. Anderson, Visual membrane: Specificity of fatty acid precursors for the electrical response to illumination. *Science* **188**, 1312-1314 (1975).

Winkler, B.S., M. E. Boulton, J. D. Gottsch, and P. Sternberg, Oxidative damage and age-related macular degeneration. *Mol. Vis.* **5**, 32 (1999).