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Light damage induced changes in mouse retinal gene expression

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Abstract

Oxidative stress plays a role in the light damage model of retinal degeneration as well as in age-related macular degeneration. The purpose of this study is to identify retinal genes induced by acute photo-oxidative stress, which may function as mediators of apoptosis or as survival factors. To accomplish this, Balb/c mice were exposed to bright cool white fluorescent light for 7 hr. Retinas were then isolated for total RNA preparation followed by Affymetrix DNA microarray analysis to compare gene expression in light damaged mice to unexposed controls. Three independent light damage experiments were carried out and statistical filters were applied to detect genes with expression changes averaging at least two-fold. Quantitative PCR was carried out to confirm altered gene expression. Seventy genes were upregulated at least two-fold immediately following light damage. QPCR confirmed upregulation of all 10 genes tested. The upregulated genes fall into several categories including antioxidants: ceruloplasmin, metallothionein, and heme oxygenase; antiapoptotic gene: bag3, chloride channels: clic1 and clic4; transcription factors: c-fos, fra1, junB, stat1, krox-24 and c/ebp; secreted signaling molecules: chitinase 3-like protein 1 and osteopontin; inflammation related genes: MCP-1 and ICAM1 and others. Upregulation of five interferon-gamma responsive genes suggests elevated interferon levels after light damage. Upregulation of three components of the AP-1 transcription factor is consistent with previous evidence implicating AP-1 in light damage pathogenesis. Four copper or iron binding proteins were upregulated, suggesting that photo-oxidative stress may affect metal homeostasis. The genes found upregulated by light damage may affect the survival of photoreceptors subjected to photo-oxidative stress.

Keywords: photooxidation; antioxidants; age-related macular degeneration; retina; gene expression; apoptosis

1. Introduction

Light damage in mice has long been used as a model system to study retinal degeneration (Noell et al., 1966). In this model and many others, photoreceptor death occurs through apoptosis, as determined by TUNEL and agarose gel electrophoresis demonstrating apoptosis-specific DNA laddering (Portera-Cailliau et al., 1994). Consistent with these results, in our Balb/c mouse light damage model, many photoreceptor nuclei label with TUNEL after a 7 hr bright cool white fluorescent light exposure (Chen et al., 2003).

The action spectrum of retinal light damage is similar to the absorption spectrum of rhodopsin (~500 nm maximum), suggesting that damage may be initiated by rhodopsin bleaching (Williams and Howell, 1983). Supporting this assertion, rodents deficient in dietary vitamin A or retinal retinoid cycle proteins (Saari et al., 2001; Sieving et al., 2001; Wenzel et al., 2001b) are less susceptible to light damage than controls. Bright light induces light damage in a transducin-independent manner, while light damage from exposure to less intense light is transducin dependent (Hao et al., 2002). The bright light induced apoptosis is dependent upon activation of the transcription factor c-fos, a component of AP-1. This c-fos upregulation plays a critical pro-apoptotic role, as the c-fos knockout mouse shows marked resistance to light damage (Wenzel et al., 2000). Further, activation of the glucocorticoid receptor, which inhibits AP-1, also protects against light damage (Wenzel et al., 2001a).

Photo-oxidative stress has been implicated in light damage pathogenesis. Immunohistochemistry has

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demonstrated labeling for markers of oxidative damage (Tanito et al., 2002). Several antioxidant genes are upregulated following photic injury, including heme oxygenase (Kutty et al., 1995), thioredoxin (Tanito et al., 2002), glutathione peroxidase (Ohira et al., 2003) and ceruloplasmin (Chen et al., 2003). Further, exogenous antioxidants protect the rodent retina from photic injury (Li et al., 1985; Noell et al., 1987).

To increase understanding of retinal responses to photo-oxidative stress, both protective and proapoptotic, we used DNA microarray analysis to examine changes in the expression of thousands of genes simultaneously (Schena et al., 1995; DeRisi et al., 1996; Farjo et al., 2002). A similar approach has recently been used to study gene expression relevant to glaucoma (Spector et al., 2002; Lo et al., 2003; Miyahara et al., 2003).

2. Materials and methods

Mice used in the experiments presented in this study were handled in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and experiments were approved by the University of Pennsylvania IRB.

2.1. Mouse photic injury

For each experiment, 10-week-old male Balb/c mice (Jackson Laboratories, Bar Harbor, ME, USA), reared with a 12 hr light-dark cycle, were divided into bright light exposure (BLE) and control groups. Control mice were dark adapted for 24 hr and then exposed to room light (200 lux) for 7 hr before euthanasia. The BLE groups were dark adapted for 24 hr and then exposed to 10 000 lux cool white fluorescent light in a well ventilated, air conditioned room for 7 hr from 2 to 9 am. The mice were housed in clear plexiglass cages, free roaming and with free access to food and water. Outside the cages, arrayed on the top and sides were 16 4 ft-long 40 W fluorescent lights (F4ORES, General Electric, Cleveland, OH, USA). Mice were sacrificed immediately after photic injury ended. Three retinas from each group were pooled and used for RNA preparation (for microarray analysis and QPCR). Eyes from mice undergoing BLE in parallel were used for histology and TUNEL labeling to confirm light damage. Three independent photic injury experiments were performed, each with a BLE group and a control group.

2.2. TUNEL labeling to confirm light damage in each experiment

Twenty-eight hours after the end of light damage, eyes were marked for orientation by cautery at the limbus, enucleated and immersion fixed in 4% paraformaldehyde for 24 hr. Eye cups were generated by removing the anterior segment. Eye cups were cryoprotected with 30% sucrose overnight, then embedded in Tissue-Tek OCT (Sakura Finetek, USA, Torrance, CA, USA). Ten micrometers frozen sections were cut in the vertical sagittal plane through the optic nerve head. The TUNEL in situ apoptosis detection kit (Roche, Mannheim, Germany) was applied to detect cleaved DNA in the frozen sections with slight modifications to the manufacturer's protocol (Dunaief et al., 2002). TUNEL positive photoreceptor nuclei were detected within each replicate of the experiment, as previously described (Chen et al., 2003).

2.3. Histology

Seven days after light damage eyes were marked by limbal cautery, enucleated and immersion fixed in 2% paraformaldehyde, 2% glutaraldehyde in 0·1 M phosphate buffer for 24 hr. Eyes were embedded in paraffin and 5 μm thick sections were cut in the sagittal plane. Sections were stained with hematoxylin and eosin. Sections were analysed by fluorescence microscopy (for TUNEL) and brightfield microscopy (for H and E) with a Nikon TE-300 microscope (Nikon, Japan) and SpotRT Slider camera (Diagnostic Instruments, Inc., Sterling Heights, MI) with ImagePro Plus software, version 4.1 (Media Cybernetics, Silver Spring, MD, USA).

2.4. DNA microarray hybridization

For each of the three light damage experiments, three retinas from the BLE group were isolated and placed in a microfuge tube on ice. Three control retinas were placed in a second microfuge tube. In one experiment, an extra pool of three control retinas was also collected. For each pool, total RNA was isolated with TRIzol (Gibco, BRL Life Technologies, Rockville, MD, USA) and 10 μg was used for cDNA synthesis with the Superscript system (Gibco, BRL Life Technologies, Rockville, MD, USA) using T7-(dT)24 oligomer as the primer. One-third of the cDNA was saved as template for subsequent QPCR experiments. The other two-thirds were used as template for in vitro transcription (IVT) to synthesize the target cRNA. Biotin-labeled cRNA was generated using biotinylated nucleotides (PerkinElmer/NEN, Wellesley, MA, USA) with the MEGA-script T7 kit (Ambion, Austin, TX, USA), and then fragmented. Ten micrograms of the fragmented cRNA, the target sample, was hybridized to a probe array, the Affymetrix Murine Genome U74Av2 GeneChip (Affymetrix, Santa Clara, CA, USA). This array chip represents all sequences (about 6000) in the Mouse UniGene database (Build 74) that encode known genes, in addition to 6000 EST sequences. Additional annotation describing the sequences represented on the chip is available at www.affymetrix.com. Target binding was detected by staining with a fluorescent dye coupled to streptavidin. Signal intensities were recorded with an Agilent confocal laser

scanner according to the procedures developed by Affymetrix.

2.5. Data analysis

After hybridization, the microarray data were analzed in Affymetrix Microarray Suite (MAS v5.0, Affymetrix Inc., Santa Clara, CA, USA). Gene signal values were transferred to S + Array Analyzer v 1.14, Insightful Corp., Seattle WA, USA). A multi-step algorithm was implemented to select retinal genes induced upon photic injury. Signal data was normalized by the median inter-quartile range, and analysed for differential expression using Local Pooled Error (Jain et al., 2003) with a 3% False Discovery Rate (Hochberg and Benjamini, 1990). This list was transferred to GeneSpring software (Silicon Genetics, Redwood, CA, USA) and genes were further filtered for being 'present' or 'marginally present' (as defined in MAS5) in at least three of the four control groups or two of the three BLE groups. Finally, genes were filtered for fold change greater than or equal to two. The raw p-value presented in Table 2 has not been adjusted by the multiple testing correction. The adjusted p-value (adjp) was generated using the Hochberg and Benjamini algorithm, and reflects the false discovery rate.

GeneChip quality control reports were generated for each microarray sample using Affymetrix MAS5.0, and all parameters were within accepted ranges as described by the manufacturer (see Data Analysis Fundamentals manual at http://www.affymetrix.com/support/technical/manuals.affx) and as normally observed for mouse samples in the Penn Microarray Facility. Global scaling factors ranged from 1·027 to 3·226, the number of genes per array called 'present' was 33·2–41·5%, and the ratio of signals from 3′ probe sets divided by 5′ probe sets ranged from 1·36 to 1·79 for actin and from 0·73 to 0·82 for GAPDH.

2.6. Quantitative PCR (QPCR)

The mRNA levels of selected genes were confirmed with QPCR by using the cDNAs from two control and two BLE groups as templates. Primers (Table 1) were designed with

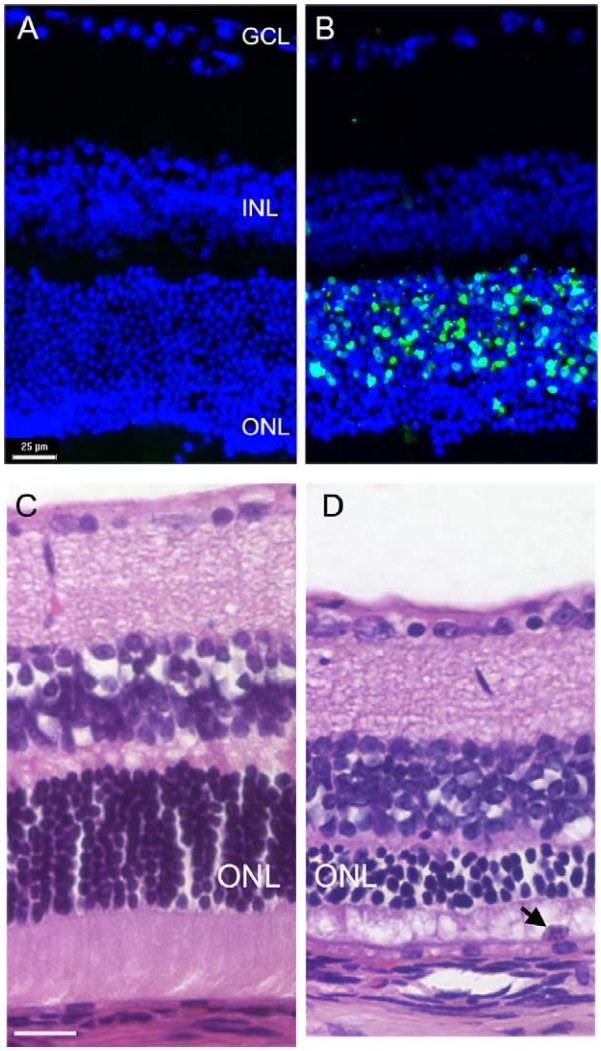

Fig. 1. Photomicrographs of TUNEL labeled or H and E stained control and BLE retinas. Twenty-eight hours after BLE, TUNEL positive photoreceptor nuclei (green) were present in the BLE retina (B) but not the control (A). All nuclei were labeled with DAPI (blue). Seven days after BLE (D), the ONL was markedly thinned compared to control retinas (C). In the BLE retinas (D) there were approximately four rows of photoreceptor nuclei remaining, and inner and outer segments were disrupted. A macrophage or abnormal RPE cell is present in the subretinal space (arrow). Abbreviations: Ganglion cell layer (GCL), inner nuclear layer (INL), outer nuclear layer-photoreceptor nuclei (ONL). Scale bar represents 25 μm.

Table 1
Primers used for QPCR

Gene name	Forward primers (5′–3′)	Reverse primer (5′–3′)
C/EBP	CCTTTGAGACTCTGAACGAC	AGTCTGTCGGAAATGTCTTTTC
Chi3L1	CCTGGCCTACTACGAGATATG	GAGCGGGAAGAATTCCTTC
CLIC4	CTTTGCCAAATTCTCTGCTTA	CGGTATTTTTTGGCCACCAC
Cp	GTAAACAAAGACAACGAGGAAT	TATTTCATTCAGCCAGACTTAG
Krox-24	GGCTCGACTGTAACTCTCAC	CGTTTTTGCTCTCAAGCCTC
Gadd45b	AGACGAAGAAGAGGAGGATG	TCTAGAGAGATATAGGGGACCC
GAPDH	TTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA
MT-II	CAACTGCTCCTGTGCCTCCG	ACGGCTTTTATTGTCAGTTACATGCTTTAT
Osmr	GAGGATACACCCAGGAACTG	AGTGTCTTTGTCTCTGAGCC
Osteopontin	TTTCCAGGTTTCTGATGAACAG	TTGCTTGACTATCGATCACATC
STAT1	GCAGTTTTCATCAGTCACCAAG	CTGAATCTAAGCAGGAACGTC

Table 2
Retinal genes upregulated by light damage detected by microarray analysis

Common	Description	Fold	Systematic	rawp	adjp	Genbank
	Antioxidants					
Mt1	metallothionein 1	6·9	93573_at	$6{\cdot}13 \times 10^{-12}$	$6{\cdot}92 \times 10^{-09}$	V00835
Mt2	metallothionein 2	9·5	101561_at	$3{\cdot}5 \times 10^{-18}$	$1{\cdot}09 \times 10^{-14}$	K02236
Cp	ceruloplasmin	5·5	92851_at	$6{\cdot}47 \times 10^{-15}$	$1{\cdot}34 \times 10^{-11}$	U49430
HO-1	heme oxygenase	3·5	160101_at	$6{\cdot}4 \times 10^{-06}$	0·001348	X56824
	Iron transport					
Lcn2	lipocalin	5·2	160564_at	$5{\cdot}43 \times 10^{-06}$	0·001182	X81627
	Pro-apoptotic					
Phlda1	pleckstrin homology-like domain	6·0	160829_at	$4{\cdot}39 \times 10^{-05}$	0·007182	U44088
	Anti-apoptotic					
Bag3	Bcl2-associated athanogene 3	10·6	161980_f_at	$6{\cdot}92 \times 10^{-07}$	0·000221	AV373612
	Inflammation					
Ifi202a	interferon activated gene 202A	6·1	94774_at	0·000223	0·026226	M31418
Ifi204	interferon activated gene 204	15·2	98465_f_at	$2{\cdot}15 \times 10^{-10}$	$1{\cdot}9 \times 10^{-07}$	M31419
Cxcl1	chemokine (C-X-C motif) ligand 1	17·2	95349_g_at	$6{\cdot}54 \times 10^{-14}$	$9{\cdot}02 \times 10^{-11}$	J04596
Cxcl2	chemokine (C-X-C motif) ligand 2	6·0	101160_at	$4{\cdot}15 \times 10^{-06}$	0·000954	X53798
MCP-1	macrophage chemoattractant 1	38·9	102736_at	$1{\cdot}15 \times 10^{-22}$	$7{\cdot}15 \times 10^{-19}$	M19681
MCP-3	macrophage chemoattractant 3	4·7	94761_at	$8{\cdot}5 \times 10^{-06}$	0·001695	X70058
Gbp1	guanylate nucleotide binding prot.	83	95974_at	$2{\cdot}91 \times 10^{-19}$	$1{\cdot}21 \times 10^{-15}$	M55544
Gbp2	guanylate nucleotide binding prot.	23	104597_at	$6{\cdot}2 \times 10^{-10}$	$4{\cdot}82 \times 10^{-07}$	AJ007970
Gbp3	guanylate nucleotide binding prot.	3·6	103202_at	$2{\cdot}68 \times 10^{-06}$	0·00068	AW047476
Chi3l1	chitinase 3-like 1	8·5	99952_at	$9{\cdot}7 \times 10^{-09}$	$5{\cdot}61 \times 10^{-06}$	X93035
B2m	beta-2 microglobulin	2·7	93088_at	$1{\cdot}99 \times 10^{-05}$	0·003435	X01838
Osmr	oncostatin receptor	7·9	102255_at	$1{\cdot}86 \times 10^{-12}$	$2{\cdot}31 \times 10^{-09}$	AB015978
Tnfaip6	TNF alpha induced protein 6	9·3	98474_r_at	$2{\cdot}8 \times 10^{-06}$	0·000694	U83903
CD44	CD44 antigen	7·0	103005_s_at	$1{\cdot}1 \times 10^{-06}$	0·000342	X66084
Icam1	intercellular adhesion molecule 1	10·1	96752_at	$3{\cdot}97 \times 10^{-07}$	0·000154	M90551
	Transcription factors					
Tieg1	TGFB inducible early growth	5·6	99603_g_at	0·000185	0·022782	AF064088
Ets2	E26 avian leukemia oncogene 2	5·5	94246_at	$4{\cdot}08 \times 10^{-05}$	0·006766	J04103
Stat1	signl. transducer, activator of txn.	3·9	101465_at	$7{\cdot}24 \times 10^{-06}$	0·001499	U06924
Junb	Jun-B oncogene	8·0	102362_i_at	$3{\cdot}01 \times 10^{-11}$	$3{\cdot}1 \times 10^{-08}$	U20735
c-fos	c-fos oncogene	9·0	160901_at	$7{\cdot}47 \times 10^{-17}$	$1{\cdot}86 \times 10^{-13}$	V00727
Gadd45b	growth arrest, DNA-damage	23·7	161666_f_at	$3{\cdot}37 \times 10^{-14}$	$5{\cdot}23 \times 10^{-11}$	AV138783
Fra1	fos-like antigen 1	15·8	99835_at	$3{\cdot}24 \times 10^{-11}$	$3{\cdot}1 \times 10^{-08}$	AF017128
Cebpd	CCAAT/enhancer binding delta	11·1	160894_at	$4{\cdot}17 \times 10^{-24}$	$5{\cdot}18 \times 10^{-20}$	X61800
Ell2	RNA Polymerase II elongation	2·5	103891_i_at	$4{\cdot}94 \times 10^{-05}$	0·007771	AI197161
Krox-24	Mus musculus zinc finger protein	6·0	98579_at	$1{\cdot}76 \times 10^{-14}$	$3{\cdot}11 \times 10^{-11}$	M28845
	Secreted proteins					
A2m	alpha-2-macroglobulin	3·8	104486_at	$5{\cdot}72 \times 10^{-05}$	0·008775	AI850558
Spp1	(osteopontin) secreted phosphoprotein 1	5·1	97519_at	$1{\cdot}79 \times 10^{-07}$	$7{\cdot}67 \times 10^{-05}$	X13986
Edn2	endothelin 2	5·2	100695_at	$4{\cdot}69 \times 10^{-07}$	0·000166	X59556
Serpina3n	serine proteinase inhibitor	3·5	104374_at	$5{\cdot}18 \times 10^{-09}$	$3{\cdot}39 \times 10^{-06}$	M64086
Plat	tissue plasminogen activator	4·8	93981_at	$3{\cdot}88 \times 10^{-08}$	$1{\cdot}83 \times 10^{-05}$	J03520
	Channels					
Gja1	gap junction membrane channel	2·1	100064_f_at	$1{\cdot}06 \times 10^{-05}$	0·001991	M63801
Clic4	chloride intracellular channel 4	4·4	94255_g_at	$6{\cdot}38 \times 10^{-08}$	$2{\cdot}83 \times 10^{-05}$	AI845237
Clic1	chloride intracellular channel 1	4·3	95654_at	$3{\cdot}98 \times 10^{-08}$	$1{\cdot}83 \times 10^{-05}$	AF109905
	Structural proteins					
Myo10	myosin X	3·2	100923_at	$8{\cdot}98 \times 10^{-06}$	0·001717	AJ249706
Msn	moesin	3·3	160308_at	$4{\cdot}45 \times 10^{-06}$	0·001005	AI839417
	Enzymes					
Mthfd2	cyclohydrolase	3·0	100046_at	$8{\cdot}73 \times 10^{-06}$	0·001695	J04627
Ctsl	cathepsin L (lysosmal enzyme)	2·6	101963_at	0·000153	0·019427	X06086

(*continued on next page*)

Table 2 (*continued*)

Common	Description	Fold	Systematic	rawp	adjp	Genbank
Crym	crystallin, mu	3·2	160937_at	$4{\cdot}45 \times 10^{-07}$	0·000162	AF039391
Dusp8	dual specificity phosphatase 8	2·7	161171_at	$3{\cdot}06 \times 10^{-06}$	0·000731	AV226788
	Cell surface protein					
H2-Q2	histocompatibility 2, Q 2	2·0	102161_f_at	0·000249	0·02838	X58609
Dtr	diphtheria toxin receptor	2·5	92730_at	0·00013	0·017128	L07264
Chl1	close homolog of L1	7·0	103088_at	$5{\cdot}25 \times 10^{-07}$	0·000176	X94310
Gp38	glycoprotein 38	2·8	104469_at	$8{\cdot}64 \times 10^{-06}$	0·001695	M73748
Emp1	emp-1 gene	4·1	97426_at	$7{\cdot}91 \times 10^{-05}$	0·011555	X98471
Tm4sf7	transmembrane 4 superfamily 7	3·7	97473_at	$4{\cdot}4 \times 10^{-07}$	0·000162	AW124470
F3	coagulation factor III	8·3	97689_at	$1{\cdot}87 \times 10^{-08}$	$9{\cdot}3 \times 10^{-06}$	M26071
	Cytoplasmic signalling					
S100a6	S100 calcium binding protein A6	2·3	92770_at	$1{\cdot}82 \times 10^{-06}$	0·000527	X66449
S100a10	S100 calcium binding protein A10	2·6	92539_at	$2{\cdot}94 \times 10^{-07}$	0·000118	M16465
S100a11	S100 calcium binding protein A11	3·5	98600_at	$4{\cdot}75 \times 10^{-06}$	0·001055	U41341
Ptpn1	Protein tyrosine phosphatase	2·4	94929_at	$7{\cdot}64 \times 10^{-05}$	0·011296	M97590
Socs3	Suppressor of cytokine signaling 3	9·3	162206_f_at	$5{\cdot}22 \times 10^{-07}$	0·000176	AV374868
Akap12	A kinase (PRKA) anchor protein	3·6	95022_at	$1{\cdot}48 \times 10^{-08}$	$8{\cdot}02 \times 10^{-06}$	AB020886
Stat3	Signal transducer, activator of txn.	2·7	99100_at	$5{\cdot}27 \times 10^{-05}$	0·008188	AI837104
	Unknown function					
Zfp36l1	Zinc finger protein 36	3·3	93324_at	$1{\cdot}62 \times 10^{-05}$	0·002842	M58566
Npn3	neoplastic progression 3	4·7	102780_at	$3{\cdot}03 \times 10^{-09}$	$2{\cdot}09 \times 10^{-06}$	Z31362
Nes	cDNA clone UI-M-BH1	4·0	103549_at	$1{\cdot}93 \times 10^{-07}$	$8{\cdot}01 \times 10^{-05}$	AW061260
Nupr1	nuclear protein 1	2·8	160108_at	$2{\cdot}85 \times 10^{-06}$	0·000694	AI852641
Ifrd1	IFN-related developmental regulator	3·2	160092_at	$2{\cdot}13 \times 10^{-06}$	0·000574	V00756
Fgls-pending	fragilis	2·8	160253_at	$1{\cdot}33 \times 10^{-06}$	0·000394	AW125390
Ier2	Immediate early response 2	2·5	99109_at	$6{\cdot}02 \times 10^{-05}$	0·009117	M59821
4930422J18Rik	EST	5·0	161817_f_at	0·000244	0·028058	AV376312
D15Wsu59e	EST	2·4	95054_at	0·000467	0·047152	AI849620
1110003O22Rik	EST	3·1	96662_at	$4{\cdot}71 \times 10^{-05}$	0·007597	AI847054

The following values are provided: mean microarray signal in three light damaged retina pools relative to four normal retina pools (fold), significance of the difference between the light damaged and normal retinas, represented as both raw p-value (rawp) and the Hochberg and Benjamini algorithm adjusted p-value (adjp), affymetrix accession number (systematic), genbank accession number (Genbank).

DS gene software (Accelrys Inc., San Diego, CA, USA), spanning the intron–exon boundary to amplify the corresponding mRNAs without amplifying potentially contaminating genomic DNA. QPCR was carried out with the SYBR green PCR master mix (ABI, Foster City, CA, USA) using the ABI 7000 fluorescein PCR detection system following the manufacturer's instructions. Electrophoresis of the QPCR product was carried out to verify that a single band of the correct size had been amplified.

3. Results

3.1. Light damage

In order to determine whether light exposure caused retinal damage, TUNEL labeling was performed on BLE and control groups in each of the three experiments. While none of the photoreceptors from the control groups were TUNEL positive (Fig. 1(A)), all retinas from the BLE groups had TUNEL positive photoreceptor nuclei (Fig. 1(B)). In sections cut in the vertical sagittal plane through the optic nerve head, TUNEL positive photoreceptors were present in highest numbers in the superior hemisphere. In a parallel experiment, retinal histology was examined seven days after light damage to evaluate photoreceptor degeneration. In sections from BLE retinas, the ONL was thinned to approximately four rows of nuclei and inner and outer segments were disrupted (Fig. 1(D)). Occasional macrophages were present in the subretinal space. The ONL was thinned in approximately one-half of the retina, mainly in the superior and temporal quadrants.

3.2. Microarray identification of light damage induced gene expression changes

Having found that the BLE causes photoreceptor degeneration, we then tested for gene expression changes following BLE. Of 12 422 genes and ESTs on the Affymetrix chip, 70 showed at least a two-fold change in gene expression following BLE (Table 2). All of these genes were upregulated in the BLE group; none showed two fold

Table 3
Fold increase in expression of the indicated genes detected by QPCR in two independent light damage experiments

	Exp. 1, QPCR	Exp. 2, QPCR	Exp. 1–3, Array
Gadd45b	6·5	13·0	23·7
C/EBP	22·6	24·3	11·1
Chi3L1	4·0	22·6	8·5
Osteopontin	3·3	4·0	5·1
Cp	2·8	8·0	5·5
MT-II	22·6	12·1	9·5
Osmr	13·0	23·0	7·9
STAT1	2·6	4·0	3·9
Krox-24	4·0	11·3	6·0
CLIC4	2·5	4·0	4·4

The mean increase in expression in the three light damage experiments detected by microarray analysis is also listed. For each gene, fold increase is determined by comparing signal in light damaged retinas immediately after 7 hr of light exposure to signal in control retinas.

or more down-regulation. The degree of upregulation ranged from the two-fold cut off to 83 fold, for Gbp1, an intracellular signaling G-protein. The 70 upregulated genes included four antioxidants, an iron transporter, a pro-apoptotic gene, an anti-apoptotic gene, 15 inflammation-related genes, 10 transcription factors, three ion channels, two structural proteins, four enzymes, and seven cytoplasmic signaling proteins (Table 2). Several functionally related proteins were upregulated. These include AP-1 transcription factor proteins fos, fra1 and junB, antioxidant metallothionein 1 and 2, chemokines cxcl 1 and 2, macrophage chemoattractant proteins 1 and 3, calcium channels clic 1 and 4, large G-proteins Gbp 1,2, and 3, calcium binding proteins S100a6, a10, and a11, and interferon-activated genes 202a and 204.

3.3. Confirmatory quantitative PCR

Ten genes with upregulation ranging from 3·9 to 23·4 fold were chosen for RT-QPCR analysis. Transcript levels from a BLE retina pool were compared to those from the corresponding control pool for each of two light damage experiments. Within each light damage experiment, all ten genes showed at least two-fold upregulation in the light damage pool (Table 3). The housekeeping gene GAPDH showed no change in expression comparing BLE to controls.

4. Discussion

We used microarray analysis to compare retinal gene expression in light damaged retinas to normal retinas in three independent experiments. Seventy genes were upregulated at least two-fold immediately following light damage. Several findings support the validity of these results. First, when ten genes found to be upregulated in the microarray analysis were tested by quantitative PCR, all ten were upregulated at least two-fold in each of two independent light damage experiments. Second, two genes previously found to be upregulated following light damage, c-fos (Grimm et al., 2000) and heme oxygenase 1 (Kutty et al., 1995) were found upregulated in our study. Third, several gene families were coordinately upregulated by light damage, supporting the non-random nature of the results. No gene was down-regulated by at least two-fold, thus the acute retinal response to light damage favors gene upregulation.

While all ten genes tested by QPCR were upregulated at least two-fold in each of two independent light damage experiments, confirming microarray results, some genes showed considerable variation in the magnitude of upregulation detected by QPCR comparing experiment 1 to experiment 2. Also, the magnitude did not always correlate well between QPCR and microarray analysis. These data suggest that, while detection of two-fold or greater gene upregulation was reliable, the absolute magnitude was more experiment and animal-specific. This may result from variations in mouse behavior and thus differences in light dose during the 7 hr light exposure period. Alternatively, it is becoming clear from cross-platform transcript profiling comparisons that magnitude change is poorly correlated among various microarray platforms, QPCR and SAGE, but at least detection and direction of change is more consistent (personal communication, Microarray Symposium ABRF Annual Meeting, 2004).

Several of the genes identified as upregulated in this study have already been implicated in the pathogenesis of light damage. A member of the AP-1 transcription factor family, c-fos has previously been shown to be upregulated following light damage (Grimm et al., 2000) and is essential mediator of light damage induced apoptosis (Wenzel et al., 2000). In our study, two other members of the AP-1 family, fra-1 and junB, were also upregulated by light damage. The dimeric AP-1 transcription factor is formed by either c-fos or fra-1 binding to jun proteins, or jun proteins homodimerizing (Shaulian and Karin, 2002). Mice lacking c-fos are resistant to light damage (Grimm et al., 2000). When the fra-1 gene is ‘knocked-in’ to the fos locus, causing fra-1 to be regulated by the c-fos promoter, susceptibility to light damage was restored. Since fra-1 lacks a transactivation domain, but AP-1 complexes containing fra-1 bind the same promoter DNA sequences as c-fos containing complexes, it was postulated that fra-1 induces apoptosis by inhibiting transcription of pro-apoptotic genes. Our finding of junB upregulation is consistent with this idea, since junB can also repress some genes containing AP-1 binding sites (Hsu et al., 1993). Further indications that junB may be an important mediator of light damage are provided by experiments showing that absence of junD has no affect on light damage (Hafezi et al., 1999a) and interference with c-Jun’s phosphorylation, essential for its transactivation activity, also has no effect

on light damage (Behrens et al., 1999). Experiments using antibody interference analysis with light damaged C57BL6/129 mice did not identify junB is a major component of AP-1 complexes (Hafezi et al., 1999b). It is possible that junB is a low abundance, but functionally significant component of AP-1, or that strain differences affect AP-1 composition.

Oxidative stress has been implicated in the pathogenesis of photic injury and several genes were previously shown to be upregulated following photic injury, including the antioxidants heme oxygenase 1 (HO-1) (Kutty et al., 1995), thioredoxin (Tanito et al., 2002), and glutathione peroxidase (Ohira et al., 2003). We detected upregulation of antioxidants HO-1, ceruloplasmin (Cp), and metallothionein (MT) 1 and 2. Cp is a ferroxidase that functions as an antioxidant by oxidizing iron from its ferrous to ferric form, decreasing the amount of damaging hydroxyl radical produced by ferrous iron through its reaction with hydrogen peroxide. The assertion that iron may be involved in photo-oxidative stress is also supported by the finding of protection of rats from light damage by the iron chelator deferoxamine (Li et al., 1991). We previously reported upregulation by light damage of retinal ceruloplasmin mRNA detected by QPCR and protein detected by Western and IHC (Chen et al., 2003). Ceruloplasmin plays an important role in retinal health, as patients with aceruloplasminemia have retinal degeneration (Yamaguchi et al., 1998). While retinal histopathology of human aceruloplasminemia has not been reported, mice with mutations in both ceruloplasmin and its homolog hephaestin have massive RPE iron overload with secondary RPE degeneration (Hahn et al., 2004; submitted), suggesting that both ceruloplasmin and hephaestin play a critical role in RPE iron homeostasis. In addition to upregulation following light damage, ceruloplasmin is upregulated in the retina in a monkey glaucoma model (Miyahara et al., 2003) and hephaestin is upregulated in lens epithelial cells exposed to reactive oxygen species (Spector et al., 2002).

Metallothionein (MT), another antioxidant, is a small, copper and zinc binding protein that can quench superoxide and hydroxyl radical. In this study, both MT-1 and 2 isoforms were upregulated. In another study, we found that MT was upregulated following light damage at the protein level as determined by Western analysis and IHC (Chen et al., 2004). Previous studies have detected MT in human and rodent retina (Nishimura et al., 1991; Tate et al., 1995; Chen et al., 1999; Lu et al., 2002; Tate et al., 2002). Over-expression of MT-I in human RPE cultures protects against toxic levels of cadmium, heme- and iron-induced oxidation and UV light-induced apoptosis (Lu et al., 2002).

Fifteen inflammation-related genes were upregulated by light damage. Macrophage chemoattractant protein-1 (MCP-1) may recruit choroidal macrophages to scavenge retinal debris following light damage, as macrophages have been localized in the subretinal space following light damage (Li et al., 1991) (Fig. 1(D)). MCP-1 may also play an important role in the clearance of retinal debris in more chronic situations, as old mice lacking MCP-1 or its receptor develop thickening of Bruch's membrane and retinal degeneration (Ambati et al., 2003). Macrophage function is affected by interferon-gamma, produced by T and NK cells, and the upregulation by light damage of five interferon-gamma responsive genes (Gbp1, 2 and 3, and Ifi202a and Ifi204) suggests that interferon gamma levels increase following light damage.

Chitinase 3-like protein 1 (Chi3L1), also known as YKL-40 is a secreted glycoprotein produced by chondrocytes and macrophages, has mitogenic activity is associated with tissue remodeling and is a migration factor for vascular endothelial cells (De Ceuninck et al., 2001). It is present in the retina and the RPE/choroid complex and in surgically excised choroidal neovascular membranes from patients with AMD. It is upregulated in laser induced choroidal neovascular membranes in mice and downregulated in mouse retina by estradiol (Rakic et al., 2003). It is also upregulated in a monkey model of glaucoma (Miyahara et al., 2003). Its upregulation following photic injury suggests that it may play a role in tissue remodeling or 'wound healing' following photo-oxidative stress or retinal injury.

Bcl-2 associated athanogenes Bag-1 and Bag-3 both bind the anti-apoptotic protein Bcl-2 and, like Bcl-2, have anti-apoptotic activity (Doong et al., 2002). Bag-1 and Bcl-2 have a synergistic effect in protection of photoreceptors from degeneration induced by transgenic expression of a truncated rhodopsin (Eversole-Cire et al., 2000). Upregulation of Bag-3 by light damage suggests that it may help protect the retina from light damage.

Three members of the S100 family of calcium binding proteins, were upregulated by light damage. One related member of this family, S100a1, localizes to bovine rod outer segments and stimulates the disc membrane bound guanylate cyclase in a calcium dependent manner (Rambotti et al., 1999), suggesting that the other members of the S100a family found upregulated in this study might also modulate cGMP levels and thus phototransduction in response to BLE.

Members of the Chloride Intracellular Channel (CLIC) family of proteins have several subcellular locations. CLIC4 localizes to mitochondria and can induce apoptosis. Overexpression of CLIC4 by transient transfection reduces mitochondrial membrane potential, releases cytochrome c into the cytoplasm, activates caspases and induces apoptosis. CLIC4 is additive with Bax in apoptosis induction and antisense CLIC4 does not block Bax induced apoptosis, indicating that CLIC4 and Bax may induce mitochondria-mediated apoptosis through independent pathways (Fernandez-Salas et al., 2002). CLIC1 inserts in the nuclear membrane (Dulhunty et al., 2001) and is thought to play a role in cell division (Shorning et al., 2003). Both CLIC1 and CLIC4 can 'spill over' and insert in the plasma membrane when overexpressed, suggesting that upregulation following photic injury may increase

plasma membrane chloride conductance (Shorning et al., 2003). This could result in hyperpolarization of inner retinal neurons, making them more resistant to massive input from photoreceptors activated by bright light.

Based on the known functions of many of the above genes, it is possible to hypothesize their activities following retinal light damage. The balance of pro-apoptotic and anti-apoptotic gene product activity may ultimately determine each cell's fate. Some genes may be upregulated in non-photoreceptors through signaling from damaged photoreceptors or through direct photo-oxidation of nonphotoreceptors. The present study identifies 70 genes upregulated by light damage, many of which are likely to play important roles in the response to photo-oxidative stress.

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