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Quantitative AP-1 Gene Regulation by Oxidative Stress in the Human Retinal Pigment Epithelium

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ABSTRACT

The purpose of this study was to characterize the early molecular responses to quantified levels of oxidative stress (OS) in the human retinal pigment epithelium (RPE). Confluent ARPE-19 cells were cultured for 3 days in defined medium to stabilize gene expression. The cells were exposed to varying levels of OS (0–500 μM H_2O_2) for 1–8 h and gene expression was followed for up to 24-h after OS. Using real-time qPCR, we quantified the expression of immediate early genes from the AP-1 transcription factor family and other genes involved in regulating the redox status of the cells. Significant and quantitative changes were seen in the expression of six AP-1 transcription factor genes, *FosB*, *c-Fos*, *Fra-1*, *c-Jun*, *JunB*, and *ATF3* from 1–8 h following OS. The peak level of induced transcription from OS varied from 2- to 128-fold over the first 4 h, depending on the gene and magnitude of OS. Increased transcription at higher levels of OS was also seen for up to 8-h for some of these genes. Protein translation was examined for 24-h following OS using Western blotting methods, and compared to the qPCR responses. We identified six AP-1 family genes that demonstrate quantitative upregulation of expression in response to OS. Two distinct types of quantifiable OS-specific responses were observed; dose-dependent responses, and threshold responses. Our studies show that different levels of OS can regulate the expression of AP-1 transcription factors quantitatively in the human RPE in vitro. J. Cell. Biochem. 108: 1280–1291, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: GENE EXPRESSION; GENE REGULATION; AP-1 TRANSCRIPTION FACTORS; OXIDATIVE STRESS; PIGMENT EPITHELIUM OF EYE; RETINA; TISSUE CULTURE

The AP-1 transcription factor family consists of dimeric proteins constituted of members of the *Jun*, *Fos*, *Maf*, *Fra*, and *ATF* gene subfamilies. Regulation of AP-1 activity occurs at several levels including transcription factor activation through subunit phosphorylation, regulation of subunit transcription and post-transcriptional mRNA turnover, protein dimerization, and protein turnover [Mitchell and Tjian, 1989; Chinenov and Kerppola, 2001]. Expression and activation of AP-1 transcription factor constituent proteins and other immediate early gene (IEG) products are stimulated by many environmental and physiological factors including cytokines, UV damage, growth factors, oxidative stress (OS), and other cell stressors via diverse signaling pathways [Devary et al., 1991; Amstad et al., 1992; Luethy and Holbrook, 1992; Karin, 1995; Morris, 1995; Geller and Stone, 2003].

AP-1 protein dimers bind to regulatory elements present in the promoter and enhancer regions of many genes but their activity and targets depend upon the cell-specific context and the signaling pathways that stimulate their activation and expression [Kovary and

Abbreviations used: OS, oxidative stress; RPE, human retinal pigment epithelium; MAPK, mitogen-activated protein kinase; JNK, Jun-N-kinase; ROS, reactive oxygen species; *FosB*, FBJ murine osteosarcoma viral oncogene homolog B; *JunB*, jun B proto-oncogene; *c-Fos*, cellular Fos; *Fra-1*, Fos-related antigen-1; IEG, immediate early gene; SE, standard error; ANOVA, analysis of variance; loess, locally weighted scatterplot smoothing.

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Bravo, 1992; Li et al., 2002]. Known pathways through which OS activates AP-1 transcription include mitogen-activated protein kinase (MAPK), Jun-N-kinase (JNK), *p38*, and other secondary signaling pathways. AP-1 is a regulator of cell homeostasis and plays an important role in cell proliferation. It can be either pro- or anti-apoptotic, depending on homeostatic balance in the cell [Guyton et al., 1996; Hafezi et al., 1999; Karin and Shaulian, 2001]. Thus, the pattern of AP-1-family gene expression, and specifically the constitution of the dimeric transcription factors derived from this family of genes, and their effects may differ depending on the state of the cell, the stimulus, and the signaling pathway directing their activation. These differences direct not only the expression patterns of specific AP-1 family proteins, but also activation of downstream AP-1 targets and the consequent expression patterns and processes within the cell.

The cellular and molecular responses to OS are diverse. Reactive oxygen (ROS) and nitrogen species participate in many cellular processes as intracellular secondary messengers, and mediators of metabolism and apoptosis [Schreck and Baeuerle, 1991; Smeyne et al., 1993; Pilz et al., 1995; Cimino et al., 1997; Shaulian and Karin, 2001]. Numerous antioxidant proteins including glutathione, catalase, heme-oxygenase-1, superoxide dismutases, and thioredoxin, to name just a few, participate in direct regulation of the redox status of the cell. Other mechanisms of redox regulation include control of lipid peroxidation and inhibition of ROS generated by photic or physicochemical reactions.

Our laboratory has focused on developing methods to detect and quantify OS in the retina by characterizing early molecular responses to OS in the retinal pigment epithelium (RPE) in vitro. In these studies, we present what we believe is the first evidence for dose dependent, quantitative gene regulation by OS. We have identified six AP-1 transcription factor genes, from three different subfamilies, that demonstrate rapid and quantitative increases in transcription following OS in the RPE. We identified two distinct types of quantifiable transcriptional responses in these genes: dose-dependent and threshold responses.

MATERIALS AND METHODS

TISSUE CULTURE CONDITIONS

Human RPE cells (ARPE-19) were cultured in DMEM/F12 media supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA) plus L-glutamine, penicillin, and streptomycin in an atmosphere of humidified 95% air and 5% CO_2 at 37°C until confluent in single wells of 6-well plates. Initially, the cells were grown in DMEM/F12 with FBS to reduce the time to reach confluence. Cells were then rinsed and fed with NR-1 media (BioSource, Camarillo, CA) or REGM (Lonza, Walkersville, MD). These media are chemically defined tissue culture media supplemented with specific growth factors including EGF, insulin, hydrocortisone, and transferrin. After feeding with defined tissue media, the confluent cells were cultured for 3 days to stabilize gene expression.

We have previously shown that rinsing cells in vitro with buffered saline or media can induce profound increases in the expression of IEG transcription factors [Yang et al., 2006]. Single wells were used in covered tissue culture plates to minimize the potential effects of temperature, CO_2 fluctuations, and handling on gene expression when cells are removed from the incubator. All procedures were performed under dim red light illumination to minimize the potential influence of light on RPE gene expression. OS was induced by the addition of freshly made H_2O_2 solution to the defined media to bring the final media concentration to the desired level (from 0 to 500 μM H_2O_2). For the 1- to 4-h OS studies, the H_2O_2 was not rinsed from the culture well, rather, the media was aspirated and TRI reagent (Sigma, St. Louis, MO) was immediately added to lyse the cells and stabilize the RNA at the designated time points. For the 8- to 24-h OS studies, the H_2O_2 was rinsed from the culture well after 4-h using our published method to minimize induced gene expression [Yang et al., 2006]. Based on an MTT assay of confluent RPE cells, no cell death was detectable as a consequence of exposure to an OS of 500 μM H_2O_2 for up to 4-h (data not shown).

RNA ISOLATION

Total cellular RNA was isolated from the cells at time points 0-, 1-, 4-, 8-, and 24-h using TRI reagent as described above using the protocol recommended by the manufacturer. The RNA was cleaned of trace DNA contaminants by treatment with RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA) and the concentration was measured by NanoDrop spectrophotometry. The purified RNA was dissolved in DEPC-treated double distilled water at the concentration of 0.2 μg/μl and stored at −80°C.

PROTEIN ISOLATION

Protein was isolated from the phenol–ethanol supernatant after precipitation of DNA. Three hundred microliters of the supernatant was placed into microcentrifuge tubes and proteins precipitated by adding 3 volumes of acetone. The solution was mixed by inversion for 10–15 s and allowed to precipitate at room temperature (RT) for 10 min. The protein precipitate was centrifuged for 10 min at 4°C, the supernatant was discarded and the protein pellet was dispersed in 0.5 ml of 0.3 M guanidine hydrochloride in 95% ethanol with 2.5% glycerol. The pellet was washed with the guanidine wash solution and incubated for 10 min at RT. The pellet was centrifuged at 8,000*g* for 5 min, decanted and two additional washes were performed. A final wash was performed for 10 min at RT using 1.0 ml ethanol containing 2.5% glycerol. The alcohol was decanted and the tube inverted to dry the pellet. The pellet was gently resuspended in 200 μl of 1% SDS by pipetting and stored at −80°C. The total protein content was determined by the Bradford technique (Bio-Rad, Hercules, CA).

REAL-TIME PCR PRIMERS

We examined 19 genes for real-time PCR analysis from pilot microarray data (not shown) from the Human Genome U133 Plus 2.0 Array library (Affymetrix, Santa Clara, CA). These included FBJ murine osteosarcoma viral oncogene homolog B (*FosB*): forward, 5′-GTG TGA GCG CTT CTG CAG C-3′, reverse, 5′-CCA ATT CAA CGG CTC GCT T-3′; jun B proto-oncogene (*JunB*): forward, 5′-CCT TCC ACC TCG ACG TTT ACA-3′, reverse, 5′-AAT CGA GTC TGT TTC CAG CAG AA-3′; β-actin: forward, 5′-CAC CCT GAA GTA CCC CAT CG-3′,

reverse, 5′-TGC CAG ATT TTC TCC ATG TCG-3′. Target sequences for these genes were obtained from GeneChip array information at the NetAffx Analysis Center (http://www.affymetrix.com/analysis/index.affx). The primers (excepting those obtained from SuperArray) were designed using Primer Express® software v1.5a (ABI, Foster City, CA) and synthesized at IDT (Coralville, IA). Optimized primers for genes *ATF2*, *ATF3*, *c-Fos*, *Fra-1*, and *c-Jun*, were obtained commercially from SuperArray (Frederick, MD).

REAL-TIME PCR STUDIES

One microgram of total dissolved cellular RNA was reversed transcribed (RT) in 20 μl of reaction volume using the Reverse Transcription System following the manufacturer's recommended protocol (Promega, Madison, WI). The RT product was diluted 1:5 with DNase-free water. qPCR amplification was performed in 50 μl of buffer containing 1× SYBR® Green PCR Master mix (ABI), optimized forward and reverse qPCR primers and 5 μl of the 1:5 diluted RT product. The qPCR reaction was started at a 50°C hold for 2 min, then 95°C hold for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. The reaction was performed using the ABI PRISM® 7700 System.

STATISTICAL METHODS

We collected the data in triplicate samples from three experiments. Our previous studies demonstrated high quantitative fidelity for the expression levels in replicate tissue culture studies from experiment to experiment [Yang et al., 2006]. The standard error (SE) for each C_T data point within a given experiment averaged 0.32 across all studies (range 0.05–1.0).

Our statistical models include the experiment sequence number, that is, they account for possible systematic variation in transcription levels in the three experiments. We fit separate linear models for each gene and each comparison of the five OS-levels (50, 100, 200, 350, and 500 μM H_2O_2) with the respective transcription levels at 0 μM H_2O_2. Looking for statistical significance of any differences, this implies a total of 60 tested hypotheses (5 comparisons for each of the 6 genes for OS after 1 and 4 h). We control the family-wise error rate of these 60 tests at $\alpha = 0.05$ utilizing Holm's sequentially rejective Bonferroni procedure [Holm, 1979]. In addition, we used ANOVA to derive confidence intervals for (gene specific) pair-wise comparisons of the transcription levels associated with the six OS-levels 0, 50, 100, 200, 350, and 500 μM H_2O_2 after 4 h. For each gene we controlled the 95% family-wise confidence level for these intervals by adjusting the confidence intervals for the multiple comparisons (15 pair-wise comparisons) using Tukey's method [Kutner et al., 2005]. We used the computational platform R (v. 2.8.1) and R-package "multcomp" (v. 1.1-1) by Hothorn et al. [2008].

We focused throughout on C_T and differences in C_T (ΔC_T) as measures of transcription levels and maintained these scales in the reporting of our results. It should be kept in mind that a one unit decrease on the C_T-scale equates to a twofold *increase* in induced transcription. As a consequence, a value of, for example, $\Delta C_T = -4$ equates to a 16-fold increase in transcription. This quantitative increase is visually underrepresented in Figure 1 where the *Y*-axis is a log value scale.

WESTERN BLOTTING

Protein lysates (10 μg) were mixed with 2× Tris–glycine SDS sample buffer (Invitrogen, Carlsbad, CA) in final volume of 20 μl and heated at 85°C for 2 min. The protein was separated on a 4–12% Tris–glycine gel running at a constant 125 V for 90 min, and transferred onto an Invitrolon PVDF (0.45 μm pore) membrane (Invitrogen) running at a constant 30 V for 90 min. The membrane was incubated with primary antibodies (all Santa Cruz, CA) overnight at 4°C. Dilutions of the primary antibodies were as follows: Fra-1 mouse monoclonal IgG (1:200), β-actin mouse monoclonal IgG (1:1,000), Jun B mouse monoclonal IgG (1:1,000), ATF-3 rabbit polyclonal IgG (1:200), c-Fos rabbit polyclonal IgG (1:200), and FosB rabbit polyclonal IgG (1:200). The secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:2,000) was added and incubated for 1 h at RT. AP-1 protein labeling from cell lysates was compared to β-actin signals from the same membrane after stripping and rehybridization. Labeling was detected by chemiluminescence by addition of ECL Western blotting detection reagent solution (Amersham). The resulting bands were scanned on a Kodak gel scanner (Image Station 4000MM).

RESULTS

Fos GENE EXPRESSION

FosB. The expression of *FosB* is strongly induced in response to OS, increasing maximally over the first 4 h after the stress. At the highest level of OS, 500 μM H_2O_2, transcription of *FosB* increased 16-fold within 1 h of OS and remained elevated, increasing to ~60-fold within 4 h, at which point it was maximal. *FosB* expression declined 50% between 4- and 8-h after OS, and returned thereafter to baseline levels within 24 h when the OS was removed from the media using our published methods [Yang et al., 2006].

There was a strong, dose-dependent association between the level of OS and the induced changes in transcription as measured by qPCR (ΔC_T values) for *FosB* transcription at both the 1- and 4-h time points, relative to the no OS control (Table I). This quantitative relationship is demonstrated in three independent experiments as shown in Figure 1A. The horizontal axis shows the six OS-levels of 0, 50, 100, 200, 350, and 500 μM H_2O_2. The vertical axis shows ΔC_T values obtained by subtracting the individual C_T values from the respective mean C_T value for 0 μM H_2O_2 in each of the three experiments. These values are ordered from positive to negative on the graph since lower C_T values correspond to *increased* levels of transcription. The magnitude of fold-changes following OS is visually underrepresented in Figure 1 due to the log scale of the vertical axis. The measurements from three independent experiments are shown. The dashed loess-line is given for visual guidance only and is computed as a locally weighted regression line [Cleveland, 1979]. The statistical significance of OS-induced transcription compared to 0 μM H_2O_2 is given in Table I. Figure 2A shows confidence intervals for pair-wise differences of all OS-levels per gene (after 4 h).

Western blotting of FosB protein was performed over a period of 24 h following exposure to 500 μM H_2O_2 stress. There was a significant increase in total FosB protein evident 4 h after exposure

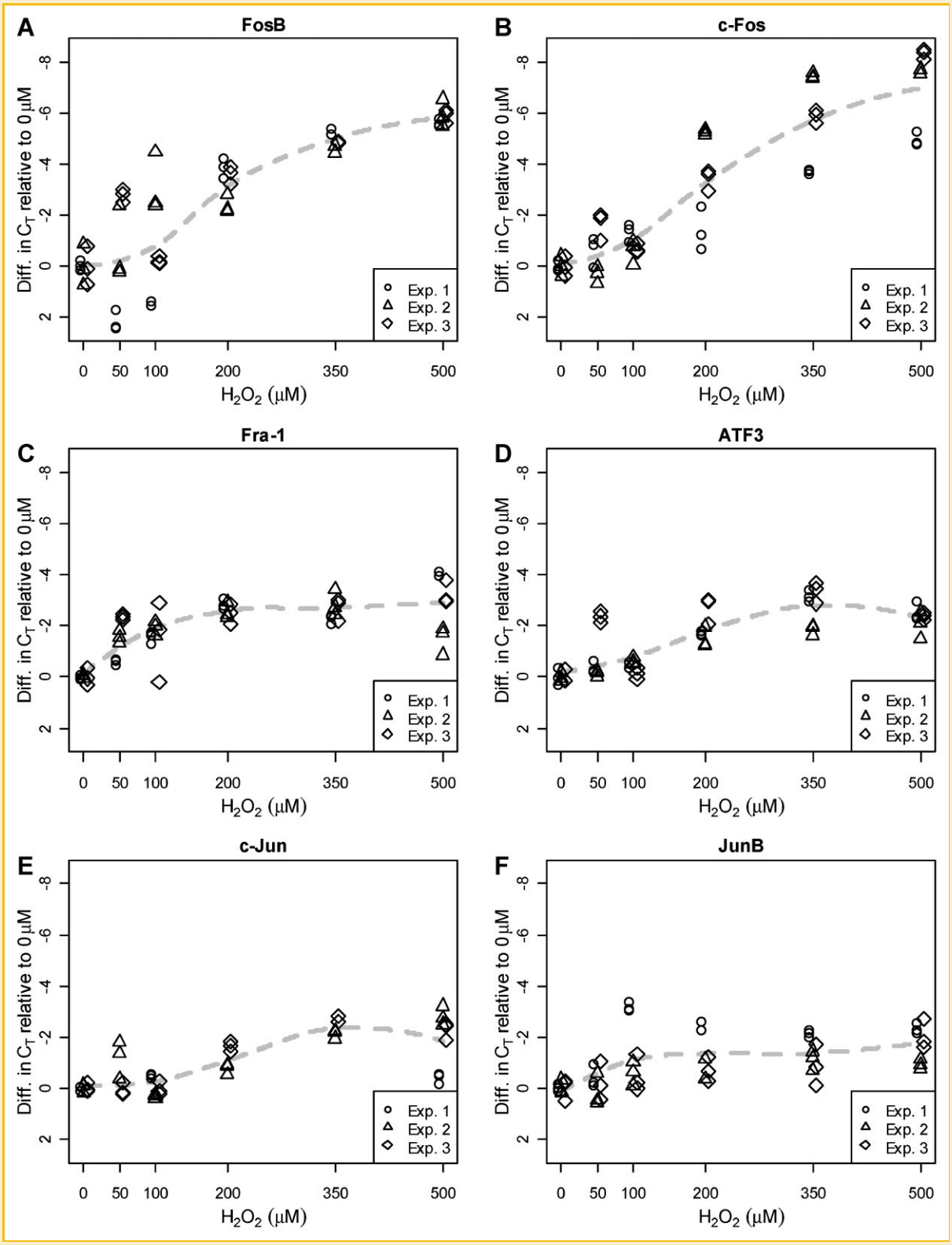

Fig. 1. Quantitative PCR analysis of AP-1 gene transcription 4 h after exposure to oxidative stress (OS). Changes in transcription were determined for (A) *FosB*, (B) *c-Fos*, (C) *Fra-1*, (D) *ATF3*, (E) *c-Jun*, and (F) *JunB* following exposure of confluent RPE cells to increasing levels of OS, from 0 to 500 µM H_2O_2 (horizontal axes). The vertical axes show ΔC_T values as obtained by subtracting the individual C_T values from the respective mean C_T value for 0 µM H_2O_2 in each of the three experiments and is log scale. These values are ordered from positive to negative since lower C_T values correspond to *increased* transcription. The magnitude of fold changes following OS is visually underrepresented due to the vertical axis log scale. The dashed loess-lines are given for visual guidance only.

TABLE I. Quantitative Induction of AP-1 Gene Transcription by Increasing Levels of Oxidative Stress

	FosB						c-Fos					
	1 h			4 h			1 h			4 h		
μM H_2O_2	ΔCt	SE	*P*-value	ΔCt	SE	*P*-value	ΔCt	SE	*P*-value	ΔCt	SE	*P*-value
50	0.63	0.23	0.0153	0.43	0.64	0.5164	1.51	0.36	0.0008*	0.64	0.29	0.0423
100	0.77	0.20	0.0017*	0.62	0.58	0.3017	2.46	0.48	0.0002*	0.88	0.17	0.0002*
200	1.79	0.26	<0.0001*	3.28	0.29	<0.0001*	3.00	0.32	<0.0001*	3.36	0.47	<0.0001*
350	1.98	0.27	<0.0001*	4.91	0.22	<0.0001*	3.38	0.31	<0.0001*	5.68	0.43	<0.0001*
500	2.42	0.36	<0.0001*	5.82	0.23	<0.0001*	3.75	0.26	<0.0001*	6.98	0.41	<0.0001*

	Fra-1						ATF3					
	1 h			4 h			1 h			4 h		
μM H_2O_2	ΔCt	SE	*P*-value	ΔCt	SE	*P*-value	ΔCt	SE	*P*-value	ΔCt	SE	*P*-value
50	0.03	0.15	0.8219	1.51	0.21	<0.0001*	0.99	0.44	0.0409	0.95	0.29	0.0049
100	0.36	0.11	0.0066	1.67	0.30	<0.0001*	1.31	0.39	0.0044	0.40	0.11	0.0027
200	1.03	0.24	0.0007*	2.64	0.12	<0.0001*	0.44	0.30	0.1599	1.95	0.20	<0.0001*
350	0.80	0.17	0.0004*	2.62	0.15	<0.0001*	0.85	0.13	<0.0001*	2.79	0.21	<0.0001*
500	0.45	0.17	0.0171	2.95	0.32	<0.0001*	0.66	0.23	0.0110	2.33	0.15	<0.0001*

	c-Jun						JunB					
	1 h			4 h			1 h			4 h		
μM H_2O_2	ΔCt	SE	*P*-value	ΔCt	SE	*P*-value	ΔCt	SE	*P*-value	ΔCt	SE	*P*-value
50	1.62	0.32	0.0003*	0.55	0.28	0.0713	0.16	0.31	0.6036	0.16	0.23	0.4882
100	1.59	0.34	0.0004*	0.03	0.11	0.7671	0.56	0.25	0.0442	1.41	0.38	0.0023
200	1.24	0.13	<0.0001*	1.20	0.16	<0.0001*	0.10	0.16	0.5582	1.23	0.27	0.0004*
350	1.02	0.37	0.0178	2.40	0.12	<0.0001*	0.04	0.22	0.8537	1.37	0.24	<0.0001*
500	0.75	0.21	0.0033	1.83	0.30	<0.0001*	0.44	0.27	0.1248	1.76	0.22	<0.0001*

ΔCt are estimated differences (with SE) of OS − control Ct values at 1- and 4-h, respectively, fitting a separate linear model for each combination of gene and OS-level that incorporates experiment ID as a factor.
P values: Marginal *P* values for ΔCt are given (two sided tests for zero difference).
*Significance as determined by Holm's sequentially rejective Bonferroni procedure that controls the family wise error rate of all shown 60 tests at $\alpha = 0.05$.

to this high level of OS, consistent with induction of *FosB* transcription over the first hour after exposure (phosphorylation of the pre-existing pool of FosB protein was not assessed due to its well know response to stress). Markedly increased levels of the total FosB protein were seen to peak at 8 h, also consistent with the time course and level of its induction after the OS. After the 8-h peak, the level of FosB protein declined, returning almost to baseline levels within 24 h (Fig. 3A).

We also determined that there was a dose-dependent relationship between the level of OS-induced *FosB* transcription and the amount of FosB protein translated after induced transcription. There is a clear interdependence between the relative level of OS-induced transcription seen in Figure 1, and the amount of protein present in the cell 8 h after OS. As the level of *FosB* transcription increases, the level of protein translated from those transcripts also increases proportionally (Fig. 3B).

c-Fos. Based upon the results seen for *FosB*, we examined the OS responses of two other AP-1 family Fos genes, cellular Fos (*c-Fos*) and the Fos-like protein (*Fra-1*). Transcriptional responses to OS were quantified using the same method 1- and 4-h after OS. The *c-Fos* gene demonstrated a similar increase in transcription to the level seen for *FosB* but rose more rapidly at 1 h, relative to *FosB*. (Table I). Transcription of *c-Fos* remained significantly elevated over control levels for up to 8 h after OS at half the maximal peak.

The strong, dose-dependent correlation between the level of OS and induced transcription seen for *FosB* were also seen for *c-Fos* (Table I and Fig. 1B). The exhibited patterns in the statistical significance of differences in transcription associated with the various OS-levels are comparable between *FosB* and *c-Fos* as can be seen by viewing Figure 2A,B side by side (but note the different scales). We examined the relationship between the dose-dependent level of OS-induced *c-Fos* transcription and the amount of c-Fos protein translated following induced transcription. There is a clear quantitative association between the relative level of OS-induced transcription following 500 μM H_2O_2 and the amount of c-Fos protein present in the cell, both 1- and 4-h after OS (Fig. 4A). The time course of c-Fos protein synthesis correlates with the induction of transcription, which is more rapid than that seen for FosB following OS (Fig. 3A).

Fra-1. The *Fra-1* gene is expressed at a higher level than *FosB* under our baseline conditions ($C_T = 21$ vs. $C_T = 26$). The pattern of *Fra-1* gene transcriptional activation in response to OS is similar to *FosB*, but the level of induced transcription is significantly less at both 1- and 4-h after OS (Table I). As with *FosB*, *Fra-1* transcription

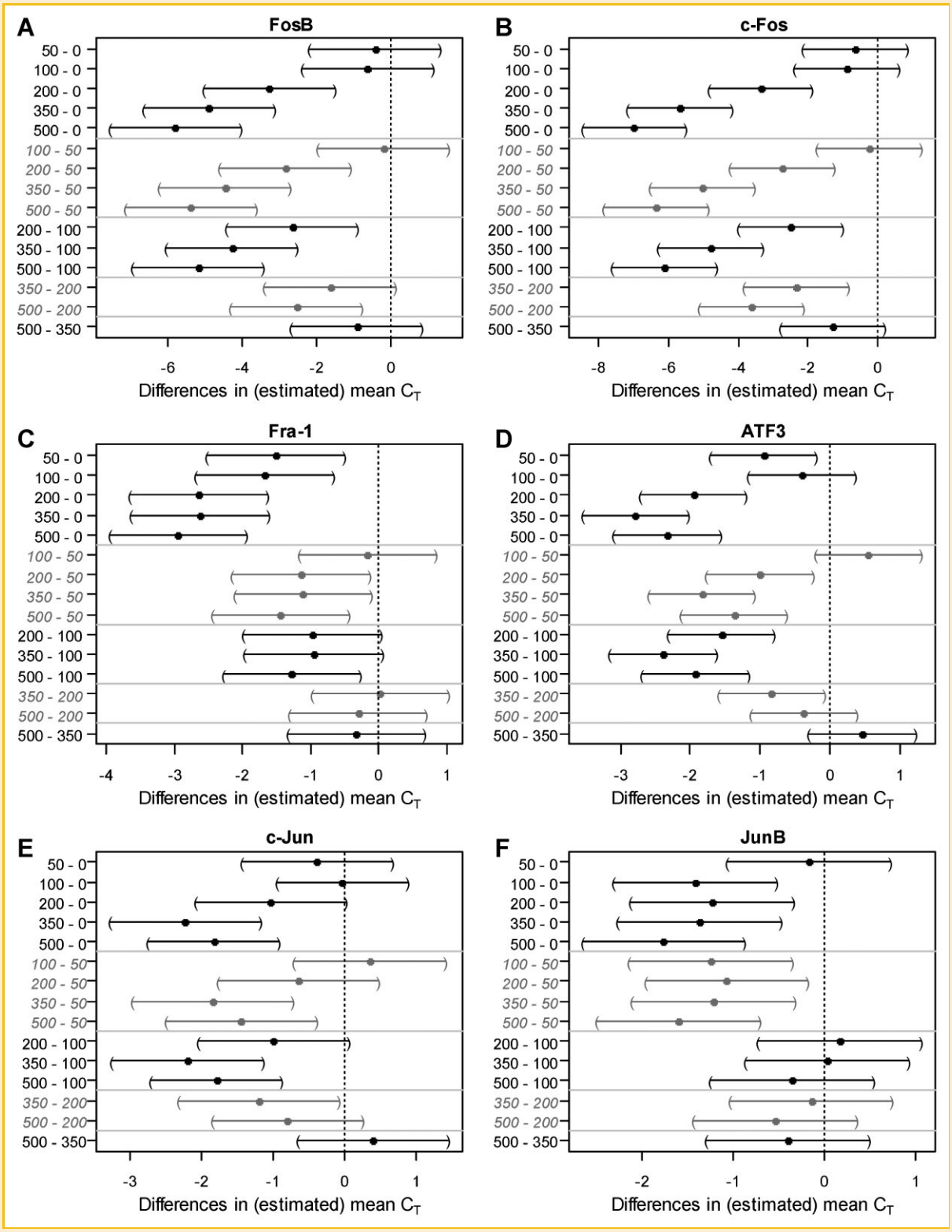

Fig. 2. Comparison of AP-1 transcription 4 h after exposure to oxidative stress (OS). Changes in transcription for (A) *FosB*, (B) *c-Fos*, (C) *Fra-1*, (D) *ATF3*, (E) *c-Jun*, and (F) *JunB* following exposure of confluent RPE cells to increasing levels of OS, from 0 to 500 μM H_2O_2. The displayed confidence intervals have a 95% family-wise confidence level and are corrected for the number of all pair-wise comparisons using Tukey's method. Point estimates of mean differences in C_T values at the various OS-levels (vertical scale) are shown as solid circles. Confidence intervals that include zero (dotted line) correspond to statistically non-significant differences in mean C_T values.

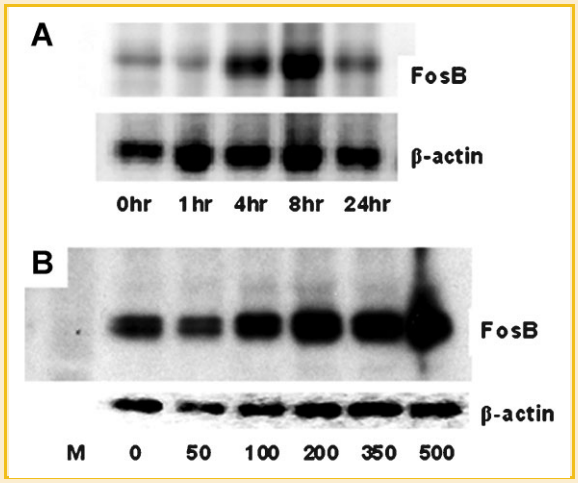

Fig. 3. Western blot analysis of FosB protein synthesis after oxidative stress (OS). A: Time course of OS-induced FosB protein synthesis over 24 h, following exposure to 500 μM H_2O_2 (compared to β-actin control). FosB protein synthesis is maximal at 8 h and returns toward baseline expression levels, but remains slightly elevated after 24 h. B: Increased synthesis of total FosB protein at 8 h in response to increasing levels of OS.

continued to increase for 4-h after OS, at which time it was maximal. As with *FosB*, expression declined significantly after 4 h and returned to near baseline levels within 8 h (data not shown).

There was a dose-dependent relationship between the level of OS and induced transcription seen for *Fra-1* but it was not as quantitative as that seen for *FosB* or *c-Fos*. We believe this was due to the lower overall level of induction (~6-fold vs. 64-fold) (Table I and Fig. 1C). Confidence intervals for pair-wise differences of transcription between the different levels of OS are shown in Figure 2C. Note the different scale for the differences in mean C_T values compared to *FosB* and *c-Fos*, indicating smaller observed differences for *Fra-1*.

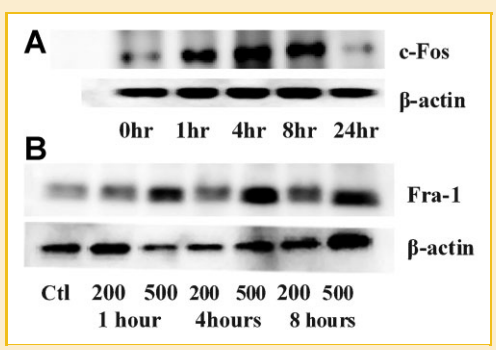

Fig. 4. Western blot analysis of c-Fos and Fra-1 protein synthesis after oxidative stress (OS). A: Time course of OS-induced c-Fos protein synthesis over 24 h, following exposure to 500 μM H_2O_2 (compared to β-actin control). Increased synthesis of c-Fos occurs earlier (1 h) and peaks earlier (4 h) than FosB, consistent with the earlier temporal increase in OS-induced transcription. B: Comparison of Fra-1 protein synthesis over 8 h at two different levels of OS, 200 and 500 μM H_2O_2. The dose-dependent difference in induction of Fra-1 protein by the two levels of OS is evident.

We examined the relationship between OS-induced *Fra-1* transcription and the amount of Fra-1 protein translated following induced transcription for two levels of OS (200 and 500 μM H_2O_2). There was also a clear quantitative correlation between the relative level of OS-induced transcription and the amount of Fra-1 protein present in the cell both 1- and 4-h after OS (Fig. 4B). The time course of Fra-1 protein synthesis correlates with the induction of transcription following OS. Fra-1 protein synthesis increases significantly for 4 h after OS, but at reduced levels for 200 μM H_2O_2 relative to 500 μM (the amount of Fra-1 protein at 500 μM/8-h is likely overestimated, compare with the β-actin signal). Fra-1 protein synthesis returned to baseline levels after 24 h (data not shown).

ATF GENE EXPRESSION

The quantitative nature of AP-1 transcription factor gene expression in OS responses in the RPE suggested to us that other genes associated with the AP-1 family complex might also show quantitative gene regulation with OS. We therefore examined the expression of two AP-1 related genes, activating transcription factors *ATF2* and *ATF3*.

High levels of OS did not increase transcription of the *ATF2* gene under our experimental conditions. No change was seen over controls at either the 1- or 4-h time points after OS (mean C_T values [with SE]: 1 h OS: Ctl 25.56 [0.06], 100 μM 25.80 [0.14], 500 μM 26.24 [0.25]; 4 h Ctl 25.96 [0.24], 100 μM 25.74 [0.23], 500 μM 26.37 [0.19]). Conversely, *ATF3* demonstrated a quantitative response at both 1- and 4-h after OS, similar to that seen for *Fra-1* (Table I). The increase in transcription was ~6-fold higher for the highest levels of OS at 4 h. There was a dose-dependent association between the level of OS and induced transcription seen for *ATF3* (Table I and Fig. 1D). Confidence intervals for the pair-wise differences of transcription between the different levels of OS are shown in Figure 2D.

We examined the relationship between the level of OS-induced *ATF3* transcription and the amount of ATF3 protein translated following 500 μM H_2O_2 OS. The time course of ATF3 protein synthesis correlates with the induction of transcription following OS. ATF3 protein synthesis increases significantly after OS, increasing to maximal levels after 8-h, and then returns toward baseline levels (though it remains elevated) after 24-h, consistent with is sustained level of transcription for at least 8 h (Fig. 5). ATF3 protein synthesis appears to be quite robust relative to the level of

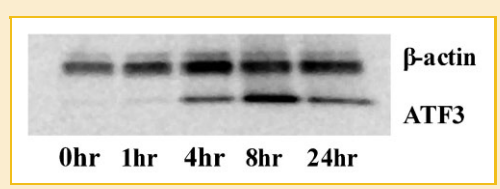

Fig. 5. Western blot analysis of ATF3 protein synthesis after oxidative stress (OS). The time course of OS-induced ATF3 protein synthesis over 24 h, following exposure to 500 μM H_2O_2 (compared to β-actin control). As with FosB, protein synthesis is maximal at 8 h and consistent with the temporal increase in OS-induced transcription. ATF3 protein declines toward baseline expression levels thereafter, but remains elevated even after 24 h.

OS-induced transcription when compared to the other AP-1 proteins examined.

Jun GENE EXPRESSION

c-Jun. The expression of *c-Jun* is moderately induced two- to four-fold in response to OS, increasing slightly over the first 4-h after stress. OS-induced *c-Jun* transcription demonstrated a threshold response at 1-h, in which the level of induced transcription was independent of the intensity of the OS (Table I).

Similarly, there was a sustained increase in *c-Jun* transcription seen at 4-h, but only at $\geq$200 μM H_2O_2. The level of transcription above this threshold did not appear to be strictly dose dependent (Table I and Fig. 1E). Confidence intervals for the pair-wise differences of transcription between the different levels of OS are shown in Figure 2E. Only when the OS-level exceeds 200 μM H_2O_2 is the associated confidence interval significantly different statistically (compare "200–0," "350–0," and "500–0").

Western blotting of c-Jun protein was performed over a period of 24 h following exposure to 500 μM H_2O_2 stress. There was a significant increase in total c-Jun protein evident 1 h after exposure to a high level of OS, all of which appeared phosphorylated as expected (Fig. 6A). The increased levels of the c-Jun protein were seen to increase and peak after 8 h, consistent with the time course and level of its induction after the OS. After the 8-h peak, the level of c-Jun protein declined over 24 h, though a significant fraction remained phosphorylated even 24 h after a 1-h OS.

JunB. *JunB* showed no significant transcriptional response at 1-h, but a threshold response was seen at 4-h for levels of OS $\geq$100 μM H_2O_2. The level of transcriptional activation at 4-h was ~2-fold and similar for all levels of OS $\geq$100 μM H_2O_2 (Table I and Fig. 1F). Confidence intervals for the pair-wise differences of transcription between the different levels of OS are shown (Fig. 2E). The dose–response is only evident at a minimum 100 μM H_2O_2 difference and is not quantitative for higher level of OS (Fig. 2F), as is seen by noting that none of the comparisons of 200, 350, or 500 μM H_2O_2 with 100 μM H_2O_2 results in statistically significant differences.

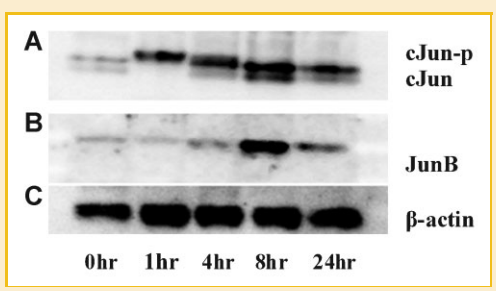

Fig. 6. Western blot analysis of c-Jun and JunB protein synthesis after oxidative stress (OS). A: Time course of OS-induced c-Jun protein synthesis (phosphorylated and unphosphorylated) over 24 h following exposure to 500 μM H_2O_2. Increased synthesis of c-Jun and activation by phosphorylation occurs early (1 h) and peaks at 8 h. Increased levels of both phosphorylated and unphosphorylated c-Jun are seen even after 24 h. B: Total JunB protein synthesis after OS is maximal at 8 h. Elevated levels are also seen after 24 h. C: β-Actin control.

We examined the relationship between the level of OS-induced *JunB* transcription and the amount of total JunB protein translated following 500 μM H_2O_2 OS. The time course of JunB protein synthesis correlates with the induction of transcription following OS. JunB protein synthesis increases significantly beginning 4-h after OS, increasing to maximal levels after 8-h, and then returning toward baseline levels after 24-h (Fig. 6B).

These results demonstrate that selected members of three AP-1 transcription factor gene families are quantitatively upregulated in a dose-dependent fashion in response to increasing levels of OS. These include: *FosB*, *c-Fos*, (and to a lesser degree, *Fra-1*); *JunB*, *c-Jun*, and *ATF3* (but not *ATF2*). Two of these genes, *c-Jun* and *JunB*, show a threshold effect, where transcription is induced by OS, but only after exceeding a threshold level.

The difference between a dose-dependent response and a threshold responses can be seen in Figure 2. Dose-dependent responses demonstrate a continued increase in transcription as OS-levels increase. This should manifest itself in a successive shift to the left of the confidence intervals when the OS-level of comparison is held fixed. *FosB* and *c-Fos* (Fig. 2A) exhibit such a pattern. The confidence intervals successively shift to the left for all estimated differences with 0 μM H_2O_2 OS and this pattern is repeated for all comparisons with 50 μM H_2O_2 OS, with 100 μM H_2O_2 OS etc. In contradistinction, *JunB* (Fig. 1F) exhibits a pattern where confidence intervals tend to have a comparable "offset" in the group of comparisons with 0 μM H_2O_2 OS, 50 μM H_2O_2 OS, etc. In addition, none of the depicted comparisons with 100 μM or higher H_2O_2 OS show significant differences, meaning that once a certain OS-level is reached, additional increase of the OS does not correspond to appreciable additional increases in transcription.

DISCUSSION

It is axiomatic that environmental stressors, including OS, have a significant impact upon IEG expression in the RPE and other cell types [Reddy and Mossman, 2002; Weigel et al., 2002; Strunnikova et al., 2004]. In our studies, we took particular care to control for many of these factors including fluctuations in light, temperature, and CO_2 content. Our recent work has shown that rinsing cells prior to isolating RNA strongly induces transcription of *FosB*, *JunB*, and other IEGs in vitro [Yang et al., 2006]. Excluding the rinse step from the short duration protocols eliminates this significant method-induced effect and permits us to isolate and examine early OS-induced responses in the RPE.

TRANSCRIPTIONAL REGULATION

These studies demonstrate that different levels of OS can quantitatively regulate the expression of AP-1 transcription factor genes and other genes modulating the cellular responses to OS. We identified two types of early transcriptional responses to OS, a dose-dependent response and a threshold response (Table II). There was a strong and dose-dependent response of *FosB* and *c-Fos* to OS. A similar but less robust dose-dependent response was seen for *Fra-1* and *ATF3*. *JunB* and *c-Jun* both showed a threshold response. For *c-Jun*, an early response to all levels of OS was seen at 1 h, but this was

TABLE II. AP-1 Gene-Specific Responses to Oxidative Stress

Dose-dependent response genes	Threshold-response genes
FosB	JunB
c-Fos	c-Jun
Fra-1	
ATF3	

sustained for 4 h only at $>100\,\mu M$ H_2O_2. *Jun B* also showed a threshold response but only after 4 h at $\geq 100\,\mu M$ H_2O_2 (Table I).

Selective upregulation of specific AP-1 family genes was seen in response to OS. *FosB* and *c-Fos* (and to a lesser degree, *Fra-1*), *c-Jun*, *JunB*, and *ATF3* were all upregulated; however, *ATF2* was not. This suggests that quantitative gene regulation by OS is gene- or promoter specific and not due simply to an overall increase in the expression of AP-1 transcription factors. Our findings confirm the opinion that *ATF2* is likely constitutively expressed.

The transcriptional responses appear to be concentration dependent or concentration gradient dependent for AP-1 genes. It appears to be independent of OS levels after induction only for *Jun*. This interpretation is supported by the fact that lower levels of OS that failed to induce the expression of *JunB* after 1-h also failed to induce expression after 4-h of OS exposure. If the stimulation of transcription were purely time dependent, we would predict that lower levels of OS that did not induce transcription after 1-h might induce gene expression over the course of 4-h exposures. This was not seen, as sub-threshold levels of OS failed to induce transcription even after 4 h.

The *Fos* and *Jun* gene families are key components of the AP-1 transcription factor complex and play a major role in directing the cellular responses to extracellular signaling by inducing diverse patterns of gene expression. These early-response genes are rapidly induced through phosphorylation of existing pools of transcription factors and activate the transcription of genes controlling both cell proliferation and apoptosis through interactions with binding sites in upstream regulatory elements [Morgan and Curran, 1991; Lazo et al., 1992; Lallemand et al., 1997]. AP-1 proteins are activated by protein kinase signaling cascades including the mitogen-activated protein kinases (MAPK/ERK, JNK, and p38), NF-κB, and others [Xanthoudakis and Curran, 1992; Cavigelli et al., 1996]. These proteins are sensitive to redox changes which affect their DNA binding activity [Fornace et al., 1988; Abate et al., 1990; Esposito et al., 1995] and thus their effect on gene transcription. It has been shown that H_2O_2 can induce AP-1 via both the JNK and p38 MAPK pathways. Multiple *cis*-acting binding sites for serum response elements, for cytoplasmic STAT target promoters and for cAMP response elements (CREs) in the *c-Fos* promoter may be activated by these kinase signaling pathways to significantly increase *c-Fos* transcription in response to OS.

TRANSLATIONAL REGULATION

The large transcriptional responses to OS seen in our cells ($\geq$60-fold for *FosB* and *c-Fos*) raised the question of whether this response was actually being translated into increased synthesis of AP-1 proteins, and the degree to which the quantitative response seen was reflected in the level of induction of AP-1 protein synthesis. Figure 3 shows that the level of FosB protein synthesized in response to OS does, in fact, reflect quantitative changes in transcription. The temporal dependence of protein synthesis shown in the Western blot appears correlated with the maximal induction of *FosB* transcription seen at 4-h after OS. In addition, there is a very strong dose-dependent correlation between the level of OS in the media and the level of FosB transcription at both 1- and 4-h after OS (Table I, Fig. 1A). The correlation exists both when compared relative to no OS controls and also between increasing levels of OS (Fig. 2A). This dose-dependent relationship is a significant finding, as is the dose dependence of induction of FosB protein synthesis (Fig. 3B). We demonstrated this relationship for the other *Fos* family genes, *c-Fos* (Table I, Figs. 1B and 4A), *Fra-1* (Table I, Figs. 1C and 4B), and also *ATF3* (Table I, Figs. Fig. 1D and 5).

Conversely, *c-Jun* and *JunB* are not upregulated in a dose-dependent manner by OS (Table I, Figs. 1E and 2E; 1F and 2F, respectively). The absence of an early response by *JunB* suggests that it is not affected by the initial pool of activated transcription factors in the cell. Rather, upregulation of this gene is a secondary event that occurs in response to new AP-1 transcription.

As expected, the rapid induction of *FosB* and *c-Fos* transcription suggests that their upregulation is controlled in part by the pre-existing pool of transcription factors that are activated (phosphorylated) in response to OS, since insufficient time has elapsed to permit a new round of protein synthesis. An early response mediated by the existing pool of dimeric AP-1 transcription factors is followed by a later response (after 1 h) that is dependent upon the synthesis of new transcription factor protein in the cell. Figures 3–6 show sustained activation and AP-1 protein synthesis following OS which is maximal at 8-h and declines toward baseline levels (but is still elevated over control levels) after 24 h. The exception to this finding is *c-Fos*, which peaks at 4 h due to its more rapid increase in transcription after OS. The temporal changes for each protein (or lack thereof) are evident in these figures.

MECHANISMS OF TRANSCRIPTIONAL REGULATION

While the exact mechanisms by which OS quantitatively regulates gene expression in the RPE remain to be elucidated, our findings show that gene regulation occurs at least in part through transcriptional regulation that is determined by the H_2O_2 concentration. Concentration-dependent regulation of gene expression by extracellular signaling has been shown to occur during embryological development. The developmental mechanism is believed to lie in a signaling diffusion gradient that activates alternative differentiation programs in subpopulations of cells within the developing embryo based upon differential receptor signaling thresholds [St. Johnston and Nusslein-Volhard, 1992]. In non-differentiating cell populations, a mechanism comprising a continuously variable transcriptional switch has been postulated [Hazzalin and Mahadevan, 2002]. In this model each round of transcription is coupled to the phosphorylated state of the transcription factors controlling activation of the gene. It predicts that instead of being "on" or "off" over a fixed interval, gene transcription is balanced by competing kinase and phosphatase

enzymes controlling the activity of transcription factors at the level of the regulatory loci.

Our findings suggest a possible mechanism of transcriptional regulation of gene expression in response to OS, that of differential dimerization, or "dimer ratio control." In this model, the relative level of AP-1 protein dimers in the nuclear and cytosolic pools differentially modulate AP-1 gene expression at each transcription factor locus through a feedback autoregulatory mechanism and may also impart specific downstream activation pathways of the classical OS-response genes. Quantitative transduction of the OS signal occurs via the dose-dependent correlation between transcription and translation of the AP-1 proteins and the temporal differences in the time course of their synthesis.

According to our hypothesis and the data presented here, the molar ratios of the individual AP-1 dimer molecules, Fos, Jun, and ATF3 will change significantly relative to stable AP-1 proteins such as ATF2 and also relative to each other during the first 8 h after OS. For example, based upon the quantitative changes in transcription, the relative ratio of c-Fos to ATF3 increases in the first 4 h after OS, but then inverts for the ensuing 24 h after a strong OS. On a molar basis, far more c-Fos heterodimers will be present in the pool of AP-1 proteins in the first 4 h after OS, whereas more ATF3 homodimers and heterodimers will be present from 8–24 h. Conversely, the ratio of FosB to ATF3 remains almost a constant throughout the first 24 h after the same OS. ATF3 homodimers act to repress ATF-responsive promoters, but ATF3 can also form heterodimers with other AP-1 proteins that are able to activate transcription. ATF3 therefore acts as either repressor or activator, depending on the context and the available pool of heterodimer protein partners. After the initial period of transcriptional activation (4 h), the increasing pool of ATF3 proteins will begin to inhibit ATF-responsive genes (including itself) through the formation of ATF3 homodimers.

Similarly, the enlarging pool of Jun:Fos heterodimers after OS will have an increased stability, DNA binding, and transcriptional activity relative to the decreasing pool of Jun:Jun homodimers. The activated Fos:Jun pools early after OS will preferentially bind to heptameric AP-1 consensus sequences (TGAGTCA) in the promoter regions of downstream targets, whereas later, Jun:ATF heterodimers will bind preferentially to the octameric cAMP-responsive elements (TGACNTCA) of other targets. We hypothesize that these differential binding efficiencies predict and direct the secondary cellular response by the classical OS-response genes such as catalase and the superoxide dismutases.

DOWNSTREAM REGULATION OF OXIDATIVE STRESS RESPONSES

A model of differential regulation of AP-1 gene activity by extracellular stimuli was proposed by Chiu et al. [1989]. Our identification of the quantitative association between the strength of the extracellular signal (in this case [H_2O_2]) and the level of induced transcription and translation provides a mechanism for this signal transduction model. Extracellular signaling leads to activation of these transcription factors via phosphorylation. The AP-1 family of heterogeneous proteins bind as homo-and/or heterodimers, to form various transcription factor complexes. The relative ratio of these discrete dimeric complexes has been shown to induce different biological responses and activate distinct biological processes within the cell [Kovary and Bravo, 1992; Chinenov and Kerppola, 2001]. Expression of the IEGs themselves is controlled through transcription factor binding to upstream *cis*-regulatory elements. Each AP-1 family gene has both common and more specific regulatory loci, some of which are also specific to signaling pathways [Hill and Treisman, 1995; Karin, 1995; Kambe et al., 1996]. It is anticipated that cellular responses to intracellular sources of OS, both chemical and photo-induced processes associated with A2E and lipofuscin, substances that accumulate with aging in the RPE, may have pathways parallel to these observed for extracellular stimuli. This will be the subject of future investigation.

How might selective upregulation of AP-1 transcription factor subfamily genes affect downstream gene activation by OS? The modified weightings of these transcribed AP-1 heterodimeric subunits also leads to differential translation, and result in changes in the relative ratios of AP-1 protein monomers and dimer complexes within the cell. Shifts in the ratios of AP-1 heterodimers have been shown to be associated with changes in cell cycle and differentiation programs [Angel et al., 1988; Halazonetis et al., 1988; Hai and Curran, 1991; Radler-Pohl et al., 1993; Marshall, 1995]. Such quantitative differences would permit qualitative transcriptional and phenotypic changes in response to OS through activation of specific patterns of secondary response genes. We hypothesize that quantitative gene regulation by OS may direct-specific downstream gene expression patterns and thus cellular phenotype by altering the ratios of specific AP-1 components available for activation of subsequent gene expression. Such a model of differential effects of signal strength on phenotype has precedence. The response of PC12 pheochromocytoma cells to growth factor stimulation (either differentiation or proliferation) depends on the signal strength, duration, or both [Dikic et al., 1994; Marshall, 1995]. Similar thresholds for transcriptional activation occur in the development of dorsoventral polarity in *Drosophila* [St. Johnston and Nusslein-Volhard, 1992]. Other regulatory mechanisms may also be at work. For example, in yeast, overexpression of the AP-1 homologue, *YAP-1*, induces resistance to OS by inducing transcription of the yeast thioredoxin (*TRX2*) via *YAP-1* binding to the *TRX2* promoter [Kuge and Jones, 1994]. The pattern of activation of classical OS-response genes such as catalase, glutathione synthetase and the superoxide dismutases may be choreographed by the specific ratios of AP-1 heterodimers generated during the early phase response described herein. We have not directly quantified AP-1 family promoter interactions with downstream OS target response genes through chromatin immunoprecipitation following OS-induced transcription. These studies are planned.

In summary, we have identified six AP-1 family genes that are either moderately or strongly upregulated rapidly in response to OS in RPE cells in vitro. The increase in transcription induced by OS is quantitative, showing dose-dependent, or threshold responses, depending on the gene. This dose-dependent response to increasing levels of OS appears to provide a mechanism by which the strength of the extracellular signal is transduced by the RPE cell through quantitative gene regulation of the AP-1 gene family.

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