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Tissue Culture Methods Can Strongly Induce Immediate Early Gene Expression in Retinal Pigment Epithelial Cells

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Abstract Expression and activation of AP-1 transcription factor proteins is stimulated by diverse physiological factors including cytokines, growth factors, and various cell stressors. The studies presented here arose out of observations in our laboratory that there was rapid and significant induction of immediate early gene (IEG) transcription in ''control'' retinal pigment epithelial cell (RPE) cultures following media changes. To clarify whether routine in vitro manipulations have the potential to induce the expression of transcription factors and non-transcription factor genes, we performed quantitative PCR studies on RPE cells in culture following various cell rinse conditions. Our studies showed that there is rapid and dramatic induction of *FosB, JunB, and EGR-1* transcription within 1 h following media aspiration and rinsing of confluent cells in vitro. The induction of these genes ranged from 32- to 256-fold following a buffered saline or media rinse. Modifying the rinse conditions and media used can eliminate this early response; however, a significant effect is still seen for *FosB* and *JunB*, 4 h after rinsing. The response was not seen with non-transcription factor genes and can be eliminated for most of the genes using a non-rinsing method. These studies demonstrate that rinsing cells in culture has the potential to profoundly affect subsequent analyses of gene expression and must be carefully controlled. J. Cell. Biochem. 98: 1560–1569, 2006.

Key words: AP-1; *FosB*; *EGR-1*; gene expression; *JunB*; pigment epithelium of eye; retina; tissue culture; transcription factors

The AP-1 transcription factor family consists of dimeric proteins made up of homodimer and heterodimer members of the *Jun*, *Fos*, *Maf*, *Fra*, and *ATF* gene subfamilies. Expression and activation of AP-1 transcription factor proteins and other immediate early genes (IEG) is stimulated by diverse physiological factors including cytokines, growth factors, UV damage, oxidative stress (OS), and other cell stressors via mitogen-activated protein kinase (MAPK), Jun-N-kinase (JNK), p38, and other secondary signaling pathways, reviewed by Karin [1995].

Regulation of AP-1 activity occurs at several levels, including protein activation through subunit phosphorylation, regulation of subunit transcription and post-transcriptional mRNA turnover, protein dimerization, and protein turnover, as reviewed by Chinenov and Kerppola [2001]. AP-1 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. For example, Tobiume et al. [2001] showed that H_2O_2 activates AP-1 primarily through a MAPK pathway protein, apoptosis signal-regulating kinase ASK1. Karin [1995] showed that the JNK pathway activates *cJun*, and Kovary and Bravo [1992] found increased synthesis of c-Fos/FosB proteins, and later Fra-1 following serum stimulation.

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AP-1 is a regulator of cell homeostasis and plays an important role in cell proliferation. However, it can also be either pro- or antiapoptotic depending on the homeostatic balance in the cell, as reviewed by Karin and Shaulian [2001]. Thus, the pattern of AP-1 family gene expression and its effects may differ depending on the state of the cell and the stimulus, which may direct subsequent expression patterns of the AP-1 family proteins and their downstream targets.

The studies presented in this article arose out of our observations from microarray and RT-PCR studies of gene expression in human retinal pigment epithelial (RPE) cells that suggested there was induction of IEG transcription in "control" cell cultures. In tissue culture studies, cells are routinely rinsed prior to isolation of RNA or proteins and after experimental interventions (to remove drugs, stressors, etc.) for temporal analyses of their physiologic effect on cells. The microarray data indicated that such manipulations themselves have the potential to introduce a strong physiological stimulus to the cultures, which could affect subsequent quantitative analyses of gene expression. In particular, we noted that AP-1 family genes *FosB* and *JunB* appeared to be strongly induced following standard tissue culture rinses. To clarify whether tissue culture rinsing methods have the potential to affect the transcription of specific classes of genes, we examined the expression of IEGs and non-transcription factor genes in confluent RPE cells using various cell rinse conditions.

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 6-well plates. The cells were initially grown in DMEM/F12 with FBS to reduce the time to reach confluence. Cells were then rinsed and fed with 3 ml of defined NR-1 media (BioSource, Camarillo, CA) as described below.

NR-1 is a chemically defined tissue culture medium supplemented with EGF, insulin, hydrocortisone, and transferrin. The NR-1 media used in our studies was not supplemented with FBS. We used defined media to minimize potential stimulating effects of unknown growth factors in FBS. After refeeding with NR-1, the cells were cultured for 3 days to stabilize gene expression in this media. T75 flasks of confluent RPE cells were fed with NR-1 media in parallel with the cells in the 6-well plates. The NR-1 media from these flasks was used for the tissue culture rinses using "conditioned" media described below to minimize the potential effects of stimulating gene expression in rinsed cells by the use of fresh media.

To model the protocol for a typical 1-h exposure to a stressor (e.g., an OS exposure), the cells were incubated for 1 h following an arbitrary "0 time point." RNA was isolated directly from the wells at each time point without rinsing ("No rinse" conditions, negative control). RPE cells exposed to 1 h of OS (500 μM H_2O_2) comprised the positive control. RNA was isolated by the No rinse method following OS and IEG expression levels were compared with those of non-stressed cells subjected to the rinse conditions described below. Non-stressed cells were rinsed and re-fed with conditioned NR-1 media for an additional 1, 4, or 24 h prior to RNA isolation, as would be performed to assess transcription following a 1-h stress. At the designated time points, the NR-1 medium was aspirated with a Pasteur pipette using vacuum suction, and TRI Reagent (Sigma, St. Louis, MO) was immediately added to lyse the cells and isolate the RNA.

Four different rinse conditions were used in the experiments: (1) "PBS rinse" with standard phosphate buffered saline (pH 7.4, 37°C); and (2) "Media rinse-1" in which FBS-free DMEM/F12 (37°C) was used to rinse the cells. For both the PBS rinse and Media rinse-1, a Pasteur pipette with vacuum suction was used to remove the media and the cells were rinsed twice and then refed with conditioned media. In (3) "Media rinse-2," FBS-free DMEM/F12 (37°C) was used to rinse the cells as above but the media was removed and the cells were rinsed using a manual pipetting method (not vacuum aspiration) with dropwise addition of media. Finally, (4) "Media replacement" was performed by rinsing the cells with conditioned NR-1 media during which half of the well volume was replaced two times by manual pipetting in a dropwise fashion.

To minimize the potential effects of temperature and CO_2 fluctuations on gene expression

when cells are removed from the incubator for rinsing, only a single well was used in each tissue culture plate. All manipulations in these studies were performed under dim red light illumination to minimize the potential influence of light on RPE cell gene expression.

RNA Isolation

Total cellular RNA was isolated from the cells at time points 0, 1, 4, or 24 h using TRI reagent as described above using the protocol recommended by the manufacturer. The RNA was cleaned of trace genomic DNA contaminants by treatment with DNA-Free (Ambion, Austin, TX) and the concentration was measured by 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.

qPCR Primers

Eight genes were chosen for real-time PCR analysis from pilot microarray data (not shown). These were selected 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′; heme oxygenase-1 (*HO-1*): forward, 5′-TTT CAC GTC TTG GTG CCT TTT-3′, reverse, 5′-CCC TCA CAA TTG CAC ATG TCA-3′; early growth response factor-1 (*EGR-1*): forward, 5′-TTT CAC GTC TTG GTG CCT TTT-3′, reverse, 5′-CCC TCA CAA TTG CAC ATG TCA-3′; catalase (*CAT*): forward, 5′-CAG GTG CGG GCA TTC TAT GT-3′, reverse, 5′-CCG GCA ATG TTC TCA CAC AG-3′; glutathione synthetase (*GSS*): forward, 5′-AAG GTC CAT GAA CCC TGC C-3′, reverse, 5′-GGC ACT GGA ACC TGC TGA AA-3′; and superoxide dismutase-2 (*SOD2*): forward, 5′-TGC TGC TTG TCC AAA TCA GG-3′, reverse, 5′-CAC ACA TCA ATC CCC AGC AGT-3′. Optimized *NF-κB* p105 primers were obtained from SuperArray (Frederick, MD).

Target sequences for these genes were obtained from GeneChip array information at NetAffx Analysis Center (http://www.affymetrix.com/analysis/index.affx). The primers (excepting *NF-κB*) were designed using Primer Express® software v1.5a (ABI, Foster City, CA) and synthesized at IDT (Coralville, IA).

Real-Time PCR

One microgram of total cellular RNA was reversed transcribed (RT) in 20 μl of reaction volume using the Reverse Transcription System (Promega, Madison, WI). The RT product was diluted 1:5 with DNase-free water and 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 qPCR was run in the ABI PRISM® 7700 Sequence Detection System. Data were analyzed using a student *t*-test with SPSS software (SPSS, Inc., Chicago, IL). All qPCR values graphed are the mean and standard deviations of triplicate samples.

Quantitative expression levels were examined for several genes in replicate studies to determine the fidelity of the quantification from experiment to experiment. A representative example is that of *JunB* expression over four independent experiments. The mean C_t values (amplification cycle thresholds) for *JunB* transcription (±SD) at 0, 1, and 4 h following a rinse were 23.01 ± 0.47, 20.27 ± 0.87, and 20.96 ± 0.97, respectively, as *JunB* expression is stimulated by rinsing. C_t values varied by less than half a cycle from experiment to experiment in stable control wells, but varied by up to one cycle when measured 4 h after stimulation. The SD within a given experiment (e.g., *JunB*) ranged from 0.05 to 0.37 cycles ($n = 6$).

Due to the inter-experimental fidelity of the data, and the large quantitative fold changes (compared to the controls) seen within experiments, replicate wells were not analyzed for every gene. Selected data sets were replicated by repeat culture and RNA analysis where indicated to clarify variances in the qPCR data. In addition, as seen for *EGR-1*, some variation in the basal levels of IEG expression at time point 0 was seen. Thus, replicate studies could not be directly compared for all genes (although absolute changes in gene expression from basal levels were compared by differences in $[\Delta C_t]$).

RESULTS

Our studies demonstrate that IEG expression is strongly and rapidly induced within 1 h by rinsing cells with PBS or media. Rinsing with conditioned media and using a method designed to minimize shifts in CO_2, temperature, and potential shear forces eliminated the transcriptional response in AP-1 genes at 1 h, but still caused a significant increase in transcription at 4 h after the rinse.

OS-Positive Control

FosB, JunB, and EGR-1 gene expression was quantified following a strong OS (500 μM H_2O_2, for 1 h) without rinsing. Each IEG was strongly induced by the OS. There was a 64- and 128-fold increase in *FosB* and *EGR-1* transcription, respectively, over 4 h after OS. *JunB* transcription increased fourfold over the same time period (Fig. 1). Remarkably, the induction of IEG expression by a strong OS was comparable to that seen following a standard cell culture rinse.

FosB Gene Expression

Quantitative *FosB* gene expression studies were performed using rinse conditions as described in the Methods. Following standard tissue culture media replacement techniques of vacuum aspiration with a Pasteur pipette followed by PBS rinse or Media rinse-1, and replacement with conditioned media, there is a 64-fold increase in *FosB* gene expression after 1 h ($\Delta C_t = 6$, or 2^6, $P < 0.001$), declining to baseline levels over 24 h (Fig. 2). Using the techniques of Media rinse-2, (manual aspiration of media with a hand pipettor and dropwise addition of media), or the partial, stepwise Media replacement (similar method), there was still a moderate though delayed, (fourfold) increase in *FosB* gene expression at 4 h ($\Delta C_t = 2$, $P < 0.001$). This method-induced effect is completely eliminated using the no-rinse technique.

JunB Gene Expression

The results of early *JunB* gene expression using rinse conditions as described were similar to those seen with *FosB*. There was a large (in this case, 16-fold) increase in *JunB* gene expression at 1 h ($\Delta C_t = 4$, $P < 0.001$), following the PBS rinse and Media rinse-1. This increase in the level of transcription declined to baseline levels 24 h after the Media rinse-1; however, unlike *FosB*, it remained elevated (eightfold) 24 h after the PBS rinse (Fig. 3).

As seen with *FosB*, there was also a significant increase in *JunB* expression (four- to eightfold, $P < 0.001$) at 4 h using the more gentle rinse techniques of Media rinse-2 and Media replacement. While the level of *JunB* expression returned to baseline 24 h after Media rinse-2, it remained elevated up to eightfold at 24 h after the Media replacement rinse ($P < 0.05$). The method-induced effect is eliminated using the no-rinse method.

EGR-1 Gene Expression

Quantitative *EGR-1* gene expression studies differed from those of *FosB* and *JunB* primarily in the intensity of the response and in the temporal aspects of the response to the rinses. As with *FosB* and *JunB*, there was a significant increase in *EGR-1* gene expression at 1 h ($\Delta C_t = 6–8$, 64- to 256-fold, $P < 0.001$), declining

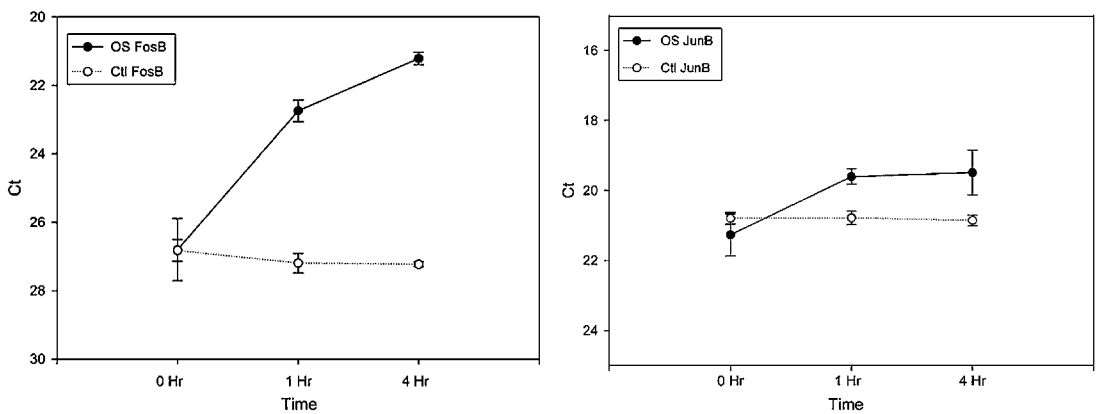

Fig. 1. Quantitative PCR analysis of *FosB* and *JunB* transcription after a strong oxidative stress (OS). Significantly increased transcription is seen for both genes at 1 and 4 h after OS (500 μM H_2O_2, for 1 h).

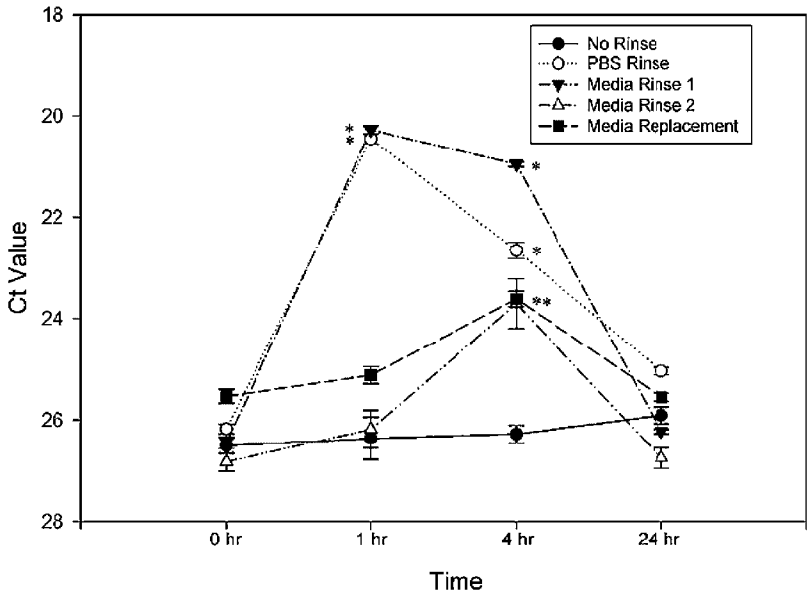

Fig. 2. Quantitative PCR analysis of *FosB* transcription 1, 4, and 24 h after rinsing under specific conditions. Significant responses were seen at 1 and 4 h after PBS rinses and Media rinse-1 ($*P < 0.001$) with maximal induction at 1 h. There was a significant increase in *FosB* transcripts 4 h after Media rinse-2 and Media replacement rinses ($*P < 0.001$) but no change in transcription was evident at 1 h. Transcription returned to baseline levels after 24 h.

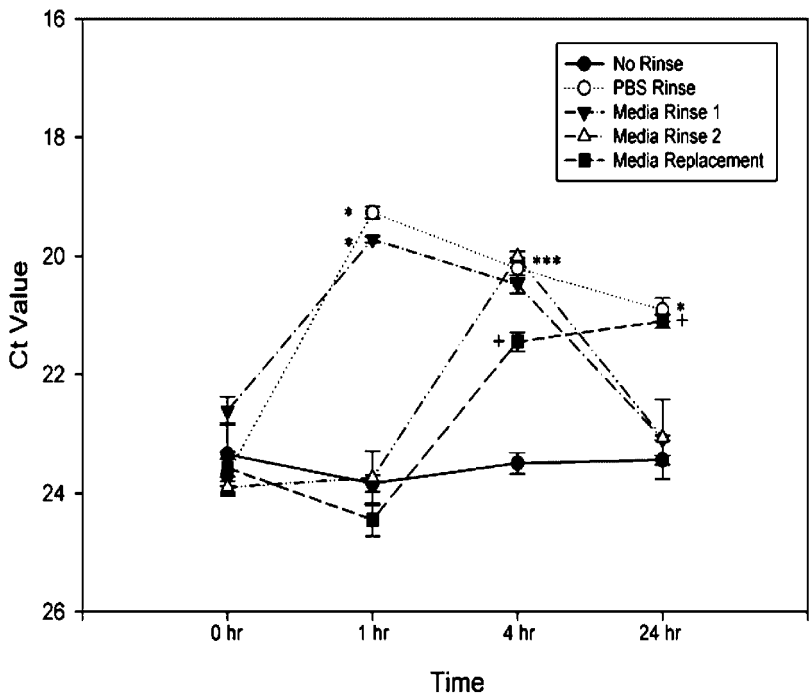

Fig. 3. Quantitative PCR analysis of *JunB* transcription 1, 4, and 24 h after rinsing under specific conditions. Significant responses were seen at 1 and 4 h after PBS rinses and Media rinse-1 ($*P < 0.001$) with maximal induction at 1 h. There was a significant increase in *JunB* transcripts 4 h after Media rinse-2 and Media replacement rinses ($*P < 0.001$, $^{+}P < 0.008$) but no change in transcription was evident at 1 h. Transcription remained elevated after 24 h following PBS and Media replacement rinses ($*P < 0.001$, $^{+}P < 0.002$).

to baseline levels over 24 h following the Media rinse-1 and PBS rinse, respectively (Fig. 4). Unlike *FosB* and *JunB*; however, the method-induced effect is seen for the techniques of Media rinse-2 and Media replacement after 1 h and is not eliminated using the no-rinse technique. Even in carefully controlled, no rinse studies, there appears to be a small methodological effect on *EGR-1* gene expression that can induce transcriptional changes. In addition, this transcriptional response occurs after 1 h and rapidly returns to baseline within 4 h for Media rinse-2. As with *JunB*, transcription of *EGR-1* following Media replacement remains elevated over baseline (twofold) even after 24 h.

The data also demonstrated that the "baseline" level of *EGR-1* transcription varied significantly in each experiment, unlike the *FosB* and *JunB* transcription factors. There was a significant difference in *EGR-1* expression at the 0 time point in the different experiments. However, the changes in *EGR-1* expression seen in response to the rinse conditions were similar in each study and followed the same temporal pattern. In addition, *EGR-1* expression returned to the original "baseline" level of each experiment (excepting the Media replacement where it remained elevated twofold).

HO-1 Gene Expression

Quantitative *HO-1* gene expression studies differed from those of *FosB*, *JunB*, and *EGR-1* in the intensity of the response and in the temporal aspects of the response to the rinses. There was no significant increase in *HO-1* gene expression after 1 h under any of the rinse conditions. However, a modest two- to fourfold increase in transcription is seen 4 h after rinsing by all methods (Fig. 5, $P < 0.05$). This effect is almost, but not quite eliminated using the no-rinse method. *HO-1* gene expression returned to baseline after 24 h.

NF-κB Gene Expression

The increase in *FosB*, *JunB*, and *EGR-1* transcription in response to the tissue culture rinse conditions suggested that this might be a non-specific response seen in different families of transcription factors. Therefore, we examined the response of NF-κB heterodimer protein p105 to the same rinse protocol. Quantitative PCR was performed 1 h after rinsing and at

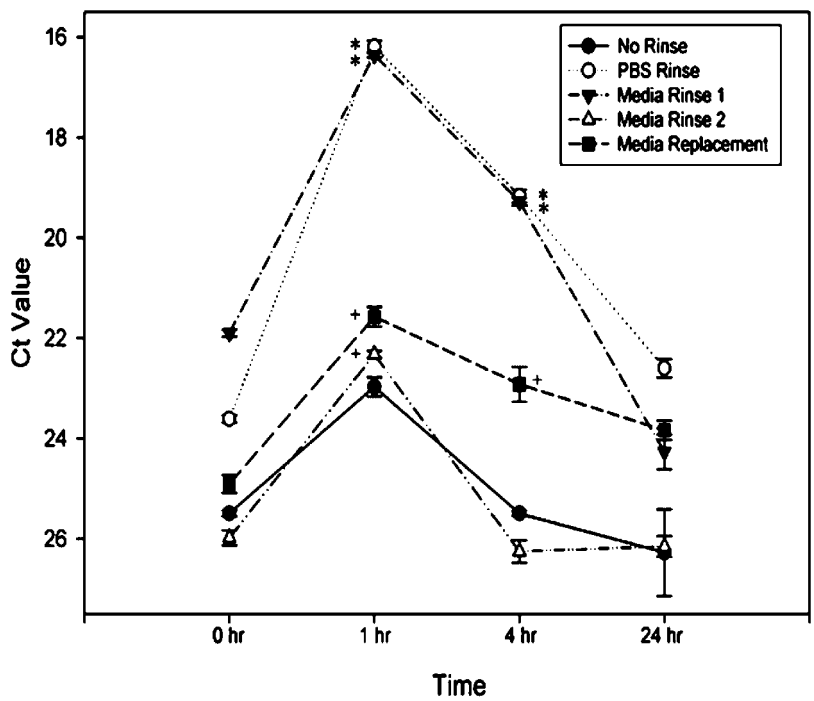

Fig. 4. Quantitative PCR analysis of *EGR-1* transcription 1, 4, and 24 h after rinsing under specific conditions. Significant responses were seen at 1 and 4 h after PBS rinses and Media rinse-1 ($^{*}P < 0.001$) with maximal induction at 1 h. Media rinse-2 and Media replacement rinses induced transcription 1 h after rinsing ($^{+}P < 0.011$, $P < 0.047$, respectively), which returned to baseline after 4 h. A similar and equivalent response was seen in the control. Transcription returned to baseline levels after 24 h.

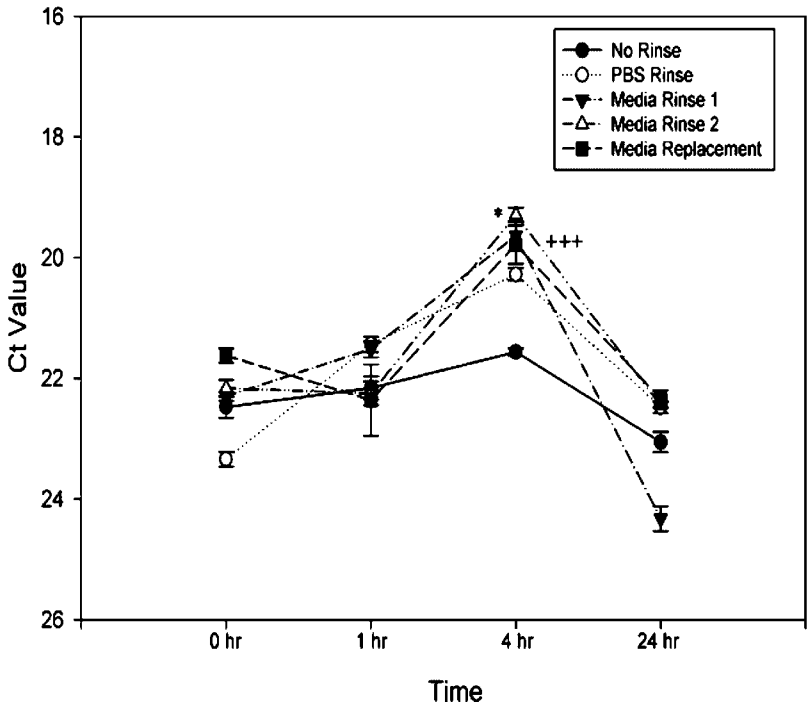

Fig. 5. Quantitative PCR analysis of Heme-oxygenase-1 (*HO-1*) transcription 1, 4, and 24 h after rinsing under specific conditions. No changes in transcription were evident at 1 h; however, a significant increase in *HO-1* transcripts was seen 4 h after rinsing using any method ($^{*}P < 0.001$, $^{+}P < 0.002$ to $P < 0.006$). Transcription returned to baseline levels after 24 h.

1 and 4 h in a separate experiment. In the first study, the ΔC_t was 0.2, 1 h after rinsing with PBS (21.9 SD $\pm$ 0.18 vs. 22.1 SD $\pm$ 0.12; P = n.s.). In the second study, the ΔC_t was 0.4 from 1 to 4 h (23.7 SD $\pm$ 0.32. vs. 24.1 SD $\pm$ 0.32; P = n.s.). Thus, there was no evident change in *p105* transcription seen following the PBS rinse conditions that stimulated the greatest transcriptional responses in the IEGs.

Other Genes

Quantitative gene expression studies were performed under the conditions described to determine if rinse-induced increases in gene expression were occurring in genes other than transcription factors. We examined three OS response genes: *CAT*, *GSS*, and *SOD2* over a period of 24 h after rinsing. There was no significant change in transcription detected in any of these genes over the first 4 h (Fig. 6). The small increase in *GSS* expression seen at 4 h (twofold) was not statistically significant due to the broad standard deviation seen for the 4 h measurements. Later quantitative measurements at 24 h demonstrated the same level of transcription as that seen at the 0 time point for all three genes (data not shown).

DISCUSSION

These studies clearly demonstrate that "routine" tissue culture methods can have previously unrecognized effects on cells in vitro and dramatically induce transcription of certain genes. Such methods, using vacuum aspiration and PBS or media rinses have the potential to induce significant IEG expression, although non-transcription factor genes can also be induced. PBS rinse and Media rinse-1 conditions dramatically increased the expression of *FosB* and *EGR-1* after 1 h by 64- to 256-fold and *JunB* by 16-fold. Even more gentle rinse conditions induced significant increases in transcription after 4 h. In most cases, the level of transcription returned to baseline within 24 h; however, *JunB* expression remained elevated and relatively stable from 4 to 24 h after the PBS and Media replacement rinses.

The potential for tissue culture manipulations to induce gene expression might be

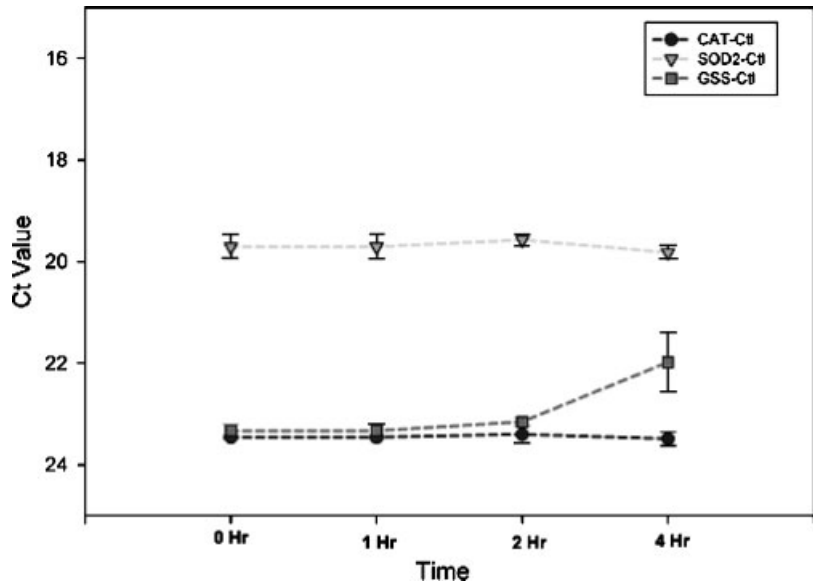

Fig. 6. Quantitative PCR analysis of Catalase (*CAT*), Glutathione synthetase (*GSS*), and Superoxide dismutase-2 (*SOD-2*) transcription 1, 2, and 4 h after rinsing with PBS. There is no statistically significant change in transcription over 4 h.

expected given the responsiveness of transcription factors in particular to changes in the extracellular environment. However, the magnitude of the effect on IEG transcription seen in our studies was quite remarkable. The level of *FosB* and *EGR-1* transcription induced by the tissue culture rinse was *equivalent* to that seen following exposure to a strong oxidative challenge (Fig. 1). Thus, experiments that attempt to quantify transcriptional responses following exposure to oxidative or other stressors, in which the stress is removed from the culture by rinsing may detect method-induced transcriptional activation, which is independent of the stress response, and may be misinterpreted in the absence of rigorous controls.

Rinsing with conditioned media and using a method designed to minimize shifts in CO_2, temperature, and potential shear forces eliminated the transcriptional response for the AP-1 genes at 1 h, but still caused a significant increase in transcription at 4 h after the rinse. Induction of gene expression was eliminated using a no-rinse method for all genes except *EGR-1* where an as yet unrecognized factor contributes to induction of gene expression at 1 h, even under the no-rinse conditions. There was no effect of rinse conditions on the transcription of non-IEGs over the first 4 h with the exception of *HO-1*, which showed a modest increase in transcription under all rinse conditions. In addition, there was no detectable increase in *NF-κB* transcription after rinsing, suggesting that the induced response has some specificity for AP-1 transcription factors and *EGR-1* and does not cause a non-specific upregulation of transcription factors in the cell.

The pattern of transiently increased expression with return to baseline levels, together with the small variances in C_t values seen in replicate samples within experiments strongly suggests that the increases in gene expression seen in response to rinsing the cells is a true biological response to the tissue culture manipulations. This interpretation was validated in replicate quantitative PCR studies of *JunB* and *FosB* (data not shown) gene expression. Thus, it appears that the very preparation of cells in vitro for subsequent analysis of RNA either by rinsing out drugs or an oxidative stressor, or simply to rinse and refeed, can induce profound changes in the transcriptional profile of the cells being examined. This occurs even under conditions in which exceptional steps are taken to minimize shifts in CO_2, pH, temperature, osmolarity, growth factors, and exposure to light.

Possible Mechanisms

There are many potential physiological stresses on cells during the process of media replacement and rinsing that are performed during routine tissue culture. These may include

mechanical, physiological, and physical stressors. For example, shear stress across the apical surface of the cells may occur when the media is aspirated and replaced. Physiologically meaningful stressors such as alterations in the extracellular pH, changes in osmolality, and transient desiccation may occur. Fluctuations in CO_2 content following removal of cells from the incubator, light stimulation, and temperature changes are other possible sources of cell stress that could induce the expression of AP-1 and other IEGs.

Previous studies by Chen et al. [2004] and others have shown that light can cause transient activation of AP-1 and other classes of genes including OS response genes and anti-apoptotic genes during light induced retinal injury in vivo. To minimize the potential influence of light on gene expression in our studies, all procedures were performed under dim red light illumination in a darkened room. We believe it is unlikely that the responses seen were due to the effects of light on the RPE cells.

The dramatic increase in IEG expression 1 h after vacuum aspiration of media (PBS and Media rinse-1) was not seen when the rinse was performed using a more gentle manual aspiration and rinse technique (Media rinse-2, Media replacement). This finding suggests the possibility that transient fluid shear stress may be inducing IEG expression. In endothelial cell studies, shear forces have been shown to activate AP-1 proteins. Earlier reports by Jalali et al. [1998], Schwachtgen et al. [1998], and Bao et al. [2000] showed that shear stress can activate both the JNK and extracellular signal-related kinase (ERK 1/2) signaling pathways This activation leads to the increased transcription of *EGR-1* as well as *HO-1*, and AP-1 genes, at least in endothelial cells as shown by Peters et al. [2002]. Although the shear stress was quantified and more sustained in these studies, a similar mechanism may be postulated for cultured RPE cells. The Media rinse-2 method uses a more gentle technique that minimizes fluid flow over the cells and the Media replacement method leaves a significant layer of fluid overlying the cells, shielding them from shear stress. Both methods serve to buffer and reduce shear forces on the cell monolayer and could account for the absence of a significant induced response at 1 h, though a delayed, but still significant increases in *FosB* and *JunB* expression was seen after 4 h even using the more gentle rinse methods. The level of IEG induction in our studies was significantly greater than that seen for endothelial cells in the studies referenced above where the increases were small (two- to threefold), suggesting that other factors may influence both early and later onset AP-1 gene expression.

It is axiomatic that changes in the extracellular environment have significant effects on IEG expression. In our studies, we took particular care to attempt to control for many of these factors including light, fluctuations in temperature, and CO_2 content (and by extension pH) by restricting removal of the culture plates from the incubator to the rinse and RNA isolation time points only. Cells were rinsed with prewarmed PBS or media at 37°C and all cells were refed with conditioned NR-1 media from confluent flasks of RPE cell cultures set up at the same time as the tissue culture plates. This protocol was established to mimic, as closely as possible, the media environment in the plates before and after rinsing. The media was conditioned by confluent RPE cells for 3 days to deplete the media of hormones and growth factors in parallel with the 6-well plate cultures, so as not to influence gene expression by the addition of fresh growth factors at the time of the rinse. The use of defined media also controlled for variability in growth factors and other unknown serum factors present in media supplemented with FBS.

Despite these controls, another possible explanation for our findings could be stimulation of the cell monolayers by the conditioned media after rinsing. Relative levels of growth factors present in the conditioned media obtained from flasks may differ from those present in the smaller wells of the tissue culture plates. Similarly, rinsing the cells with PBS or unsupplemented media followed by refeeding with conditioned NR-1 media could trigger growth factor signaling due to the relative increase in concentration of growth factors in the media compared to the rinse fluid. Lallemand et al. [1997] showed that serum stimulation stimulates the expression of *FosB* and *JunB* in human fibroblasts in vitro, and this could provide a mechanism for similar transcriptional activation seen in our studies.

Another conjecture is that the results may be due to differences in the osmolarity of the fluids used in the rinses. Although warmed

and pH adjusted, the PBS and Media rinses differed from the Media replacement fluid in that there are no proteins or lipids in the solutions. These solutes would contribute to potential differences in osmolarity between the fluids. Osmotic stress was shown to activate ERK 1/2, JNK, *p38*, and *Jak*/STAT signaling pathways in vitro in earlier studies by Gatsios et al. [1998] and Lu et al. [2000], any one of which could induce IEG transcription. Although plausible, the small differences in osmolarity between rinse fluids and their brief duration of exposure, in our view, are less likely to induce large, gene-specific increases in transcription.

SUMMARY

In summary, although rinsing cells in the course of tissue culture studies is a routine step, its impact on IEG transcription is highly significant and can induce large fold increases, particularly in *FosB*, *JunB*, and *ERG-1* transcription factors. The mechanism appears to have some specificity for AP-1 and also *ERG-1* induction and does not appear to be due to a general upregulation of transcription factor gene expression since *NF-κB* is not affected. Although the mechanism that induces transcription in these genes is not yet clear, based upon the study design, we believe that potential contributing factors include shear forces, growth factor stimulation, and possibly osmolarity shifts generated by standard rinse fluids. Future studies using a flow chamber will help to determine the role of shear stress in induced gene expression in the RPE.

These findings demonstrate that transcription factor gene expression is exquisitely sensitive to tissue culture methods and such methods can have a profound effect upon subsequent quantitative analyses of transcription. They need to be recognized to minimize the risk of method-induced error in the interpretation of results, particularly with respect to analysis of IEG expression, and they must be carefully controlled.

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