

ORIGINAL ARTICLE

Cytoprotection against thermal injury: evaluation of herbimycin A by cell viability and cDNA arrays

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ABSTRACT

Herbimycin A (HA), a known inducer of the heat shock response, was investigated for its ability to increase survival of a human cell line following thermal injury. Its effect on transcriptional activity was also assessed with cDNA arrays to provide new targets for cytoprotection. Pretreatment with at least 0.75 µg/ml HA significantly increased the fraction of cells surviving thermal injury by up to 50% (based on 8s exposure) compared to untreated controls. HA also significantly induced transcription of mRNA for HSP90 and HSP70, and protein production for HSP40 and HSP70. Gene expression profiling demonstrated that the most highly elevated genes included growth factors and transcription factors, while prominently suppressed genes included transcription factors and kinases. These results suggest that cytoprotection may be due to the contribution of the products of a significant number of genes in addition to the classic stress response genes, suggesting that modulation of these genes might induce thermotolerance and amelioration of thermal injury.

The Pharmacogenomics Journal (2002) 2, 318–326. doi:10.1038/sj.tpj.6500120

Keywords: herbimycin A; heat shock proteins; thermal injury; thermotolerance; stress response; gene expression

INTRODUCTION

Thermal injury is one of the most common forms of accidental injury resulting in 2 000 000 hospital admissions and 6500 deaths per year.¹ Also, the occurrence of high-temperature, short-duration thermal injuries, characteristic of accidental and therapeutic laser exposure, is becoming more common.^{2–4} However, there are no drug treatments currently available to salvage thermally injured cells that might accelerate the repair process by preventing loss of injured yet potentially viable cells. We previously showed that exposure to relatively high temperature (55°C) for short periods (1–8 s), as occurs during thermal injury, induced the heat shock response.⁵ The heat shock response is one of the best understood examples of cytoprotection in which a sub-lethal pretreatment leads to a significant increase in survival of cells exposed to a subsequent lethal thermal injury. Depending on temperature and length of exposure, the heat shock response can induce heat shock proteins (HSPs) that are well understood as an adaptive, protective response to heat.⁶ Under normal conditions, constitutively expressed HSPs act as molecular chaperones involved in protein folding, assembly, and transport. During stress, induced HSPs probably restore denatured proteins to their native configuration. They therefore prevent the accumulation of aggregated, misfolded, and damaged proteins.^{7–9}

The cytoprotective effects of HSPs are also being evaluated for therapeutic purposes. Aside from thermal insults, the presence of HSPs has been reported to protect against a multiplicity of injuries, many resulting from oxidative or

Received: 3 December 2001

Revised: 17 April 2002

Accepted: 18 April 2002

ischemic damage.^{10,11} A variety of other insults such as hypoxia, heavy metal, and alcohol can produce similar responses in cells. By comparing the specific pathways of induction and the resulting downstream changes in cellular function and expression, the mechanism of protection may be elucidated.

To better understand the response of human cells to sub-lethal thermal injury, we previously characterized the alterations in gene expression of ARPE-19 cells using cDNA microarrays.¹² This analysis indicated that sub-lethally injured cells induced a large number of genes including transcription factors, and those for growth regulation and DNA repair, in addition to HSPs in reaction to thermal insult.

In this study we investigated herbimycin A (HA) to determine if it is capable of inducing cytoprotection and, if so, to identify additional genes involved in cytoprotection. HA, known as a tyrosine kinase inhibitor, has been reported to activate the heat shock response^{8,13,14} and is protective against both ischemic¹³ and thermal injuries.¹⁵ It appears to cause the heat shock transcription factor-1 (HSF-1) to bind the heat shock element (HSE) leading to transcriptional activation of HSP genes.⁸ It has been shown to induce transcription of HSP70, HSP90, HSP25, and HSP30 in a number of cell types.^{8,13,14}

Since the induction of HSP cytoprotection is through gene transcription and translation, cDNA arrays were used to identify additional genes involved in cytoprotection or cytotoxicity of this compound. Gene expression profiling indicated that in addition to the induction of the classic heat shock genes (ie, HSP90, HSP70), transcription for a variety of other genes encoding growth factors and transcription factors were upregulated and may be involved in protection by HA. Therefore, HA likely exerts some of its cytoprotection through other mechanisms. By understanding how altered gene expression is involved in specific pathways of induction and the resulting downstream changes in cellular function and expression, additional mechanisms of protection may be elucidated.

RESULTS

Cell Viability

Alamar Blue™ was used to assess cell viability 24 h after heating ARPE-19 cells in a HEPES buffered saline bath for 6–8 s at 55°C. As previously demonstrated, dipping the cells attached to the coverslip into a saline bath produced a uniform injury across the cell layer with a predictable survivability that was a function of time of exposure and temperature.¹² For the ARPE cells, as used here, a temperature of 55°C for 8 s resulted in a reproducible thermal injury that when left untreated reduced cell viability to 50% compared to unheated controls (Figure 1).

Pretreatment with HA for 1 h, followed by a delay of 4 h before heating, increased cell survival in a dose-dependent manner for thermally injured cells (Figure 1). Pretreatment with concentrations of at least 0.75 µg/ml HA notably increased cell survival for all durations of heating. Under

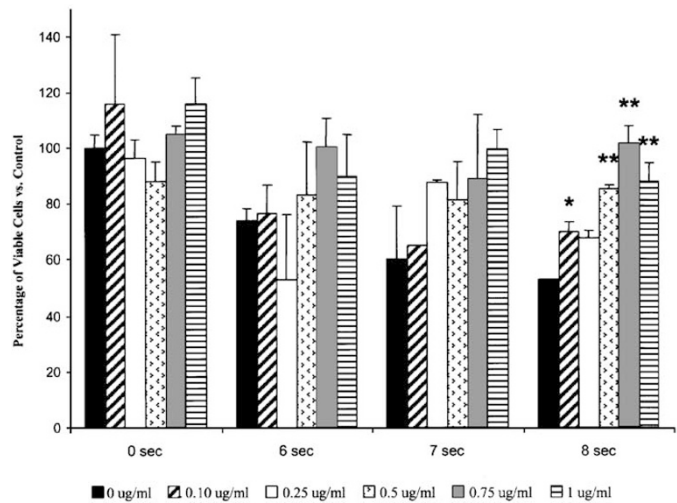


Figure 1 ARPE-19 cell viability 24 h following thermal insult at 55°C for 6–8 s ($n = 15$) with and without pretreatment with HA. Cell viability was assessed by the Alamar Blue™ fluorescence assay and expressed as a percent of heat-treated cells surviving compared with unheated control cells and cells pretreated with 0.10–1.00 µg/ml HA (* $P \leq 0.05$, ** $P \leq 0.01$ for one-way ANOVA compared to 0 µg/ml HA untreated/unheated cells, per time point).

the most severe thermal insult at 8 s, nearly all concentrations of HA (0.10 µg/ml with $P \leq 0.05$ and 0.50–1.00 µg/ml with $P \leq 0.01$) significantly increased cell survival. HA increased cell survival from 15 to 50% compared to untreated cells (Figure 1).

Gene Expression Profiling

To determine the reproducibility of cDNA array experiments, three separate array membranes were hybridized with three individual control samples. The average and standard deviation of integrated optical density (IOD) ratios between them were used to indicate the level of difference necessary to determine significance. A log-log scatter plot of three control IOD values illustrates the distribution along a line with the slope of the average intensity (Figure 2a). With an average and standard deviation of 1.10 ± 0.30 , an IOD ratio of at least 2.00 represents a significant difference ($\alpha = 0.01$). A histogram shows the distribution of IOD ratios, with a normal distribution centered at about 1.10 (Figure 2b).

To better understand the basis for HA cytoprotection and to acquire information about potential cytotoxic effects of HA, gene expression analysis with cDNA arrays was performed. Over the 24-h treatment period, 1.00 µg/ml HA significantly (≥ 2 -fold) altered transcriptional regulation of 307 of the 1176 genes on the Atlas™ Human 1.2 cDNA Expression Array membrane in unheated ARPE-19 cells (Table 1). Approximately one-fifth of the genes that were significantly elevated by 1 h remained elevated at 4 or 24 h, although typically to a lesser extent. Transcription of these genes peaked at 1 h and generally decreased over 24 h to lower or control levels. Some of the most significantly

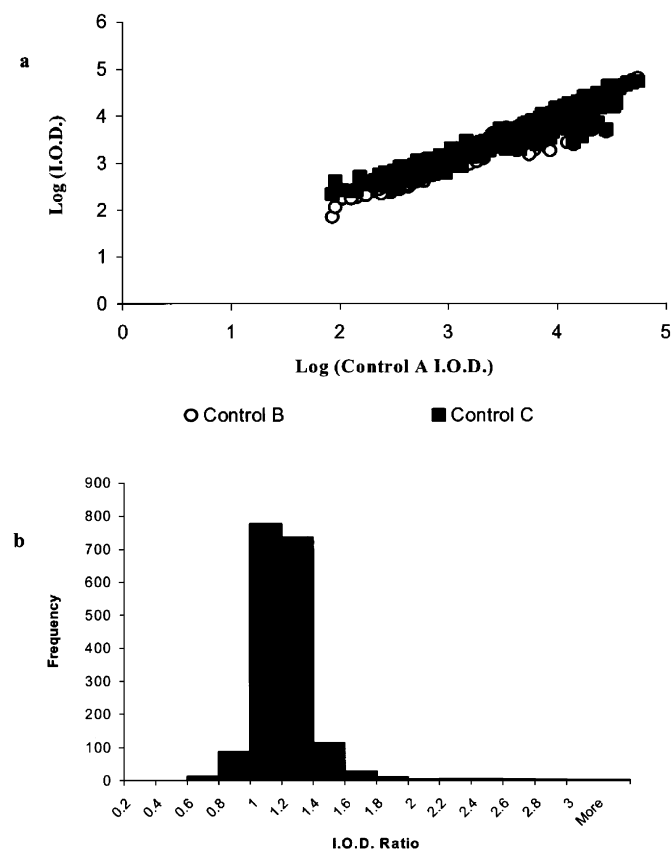


Figure 2 (a) Log-log scatter plot and (b) histogram of integrated optical densities (IOD) for three control samples from Atlas Human 1.2 cDNA Expression Arrays analyzed with AtlasImage, illustrating the reproducibility of gene expression analysis.

elevated genes at 1 h included transcription factor 3, jun D proto-oncogene, cyclin G associated kinase, endoglin, interferon regulating factor 7, macrophage receptor, and heat shock transcription factor 4. The only significantly suppressed genes were DiGeorge syndrome critical region gene DGSI, member 3 of the potassium channel subfamily K, and mucosal vascular addressin cell-adhesion molecule 1.

After 4 h of HA treatment there were 47 significantly altered genes, of which 19 were newly elevated and had not been significantly altered at 1 h. Most of these genes returned to control levels by 24 h, although several were still significantly elevated or suppressed at 24 h. Some newly upregulated genes at 4 h included runt-related transcription factor 3, HSP90, HSP70, and plasminogen activator inhibitor. After 24 h of HA treatment 57 genes were also significantly altered. Approximately one-third of these genes was newly expressed and also predominantly suppressed. The temporal pattern of expression for all altered genes was more easily evaluated through clustering. Analysis of the most highly regulated subset of genes would suggest which cellular functions are most important for the repair of injured cells. The dendrogram organized the subset of genes

(altered by ≥ 3 -fold) into groupings that were regulated in a similar manner over the 24-h time course (Figure 3).

Verification of HSP Production

HSP levels were measured over a 24-h time course following HA treatment of uninjured cells to determine the relationship between message and protein appearance. ARPE-19 cells were treated with 1.00 $\mu\text{g}/\text{ml}$ HA for 1, 4, 8, and 24 h and then evaluated over a 24-h time course for production of HSPs. Detectable increases occurred for HSP70 but not HSP40 (Figure 4a and b). HSP70 protein production was induced at 4 h, and steadily increased to nearly 2-fold the originally induced levels by 24 h.

After determining that HSP40 and HSP70 were both present 24 h following HA treatment with 1.00 $\mu\text{g}/\text{ml}$, protein levels were measured at lower concentrations of HA to determine any dose dependency. ARPE-19 cells were treated with 0.10, 0.25, 0.50, 0.75, and 1.00 $\mu\text{g}/\text{ml}$ HA for 24 h. After 24 h, cells expressed both HSP40 and HSP70 (Figure 5a and b). HSP40 was expressed constitutively, but HA did not upregulate expression at any concentration. HSP70 was induced with 0.25 $\mu\text{g}/\text{ml}$ HA, but increasing concentrations of HA did not increase protein production.

DISCUSSION

Thermal injury experiments verified that ARPE-19 cells pretreated with 0.75–1.00 $\mu\text{g}/\text{ml}$ HA were protected against lethal thermal insult at 55°C. Gene expression profiling of ARPE-19 cells treated with HA revealed a significant upregulation of mRNA for HSP70 and HSP90. Western blotting corroborated protein production for HSP40 and HSP70. The mRNA and protein results also indicated that there was a lag time between upregulation in transcription and production of HSP70 product. It is therefore conceivable that with a longer response time before thermal injury, allowing for greater HSP70 protein production, it may be possible to obtain a greater protective response. The observed significant increase in cell survival suggests that HA confers cytoprotection against thermal injury, but the low levels of HSP70 production (2.1-fold increase) suggest that other genes may also play a role in providing protection as many other genes were upregulated by over 3-fold (see Table 1 and Figure 3). It remains to be determined if post-injury treatment may provide any cytoprotection.

Furthermore, it has been suggested that HSP nuclear translocation does not always occur with HA induction,¹⁵ and that HA actually exerts its cytoprotection through other mechanisms. HA has also been shown to exert ameliorative properties following ischemic injury^{13,15} and hypoxia.¹⁶ In addition to HSP induction, or possibly in tandem, one feasible mechanism to account for the protection may be through attenuation of neovascularization.¹⁶ It has been well documented that a strong correlation exists between the inhibition of tyrosine phosphorylation and the suppression of cell proliferation.¹⁷ Induction of HSP70 in retinal microvascular endothelial cells,¹⁶ and HSP70, HSP90, and HSP25 in rat neonatal cardiomyocytes have been shown to be protective against simulated ischemia.^{13,15}

Table 1 Fold elevation or suppression of some of the most highly altered genes on the Atlas™ Human 1.2 cDNA Expression Array following 1-, 4-, and 24-h treatments with 1.00 µg/ml HA. The genes are organized from the most elevated to suppressed, beginning at 1 h through 24 h

GenBank Accession #	Protein/gene	1 h	4 h	24 h
AA026644	Transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)	5.51	5.27	
AA598659	ESTs, moderately similar to S23647 NuMA protein-human	5.46	3.08	2.36
AA131585	Jun D proto-oncogene	5.44		
AA428959	Cyclin G associated kinase	5.35	2.92	
AA446108	Endoglin (Osler-Rendu-Weber syndrome 1)	4.79	3.27	
AA443090	Interferon regulatory factor 7	4.75	2.42	2.28
AA485867	Macrophage receptor with collagenous structure	4.48	3.58	
W72250	Calcium channel, voltage-dependent, beta 1 subunit	4.46	2.74	
AA425806	Suppressin (nuclear deformed epidermal autoregulatory factor-1 (DEAF-1)-related)	4.45		
H15445	Sex gene	4.29	4.07	
H25223	Mesenchyme homeo box 2 (growth arrest-specific homeo box)	4.29		
W86182	Pinin, desmosome associated protein	4.28		
R46202	Iroquois-class homeodomain protein	4.11	2.92	2.52
AA180013	Hyaluronan-binding protein 2	3.74	2.28	
AA683219	Human immunodeficiency virus type I enhancer-binding protein 2	3.72		
AA464748	Collagen, type VI, alpha 2	3.70	2.13	
N73958	Small inducible cytokine subfamily A (Cys-Cys), member 25	3.70		2.35
T46897	Cell membrane glycoprotein, 110000 M(r) (surface antigen)	3.69	2.32	
AA504128	RAE1 (RNA export 1, <i>S.pombe</i>) homolog	3.69		2.41
AA448959	NADH dehydrogenase (ubiquinone) Fe-S protein 5 (15 kDa) (NADH-coenzyme Q reductase)	3.67	2.09	
W35411	Neuro-oncological ventral antigen 2	3.59	2.34	
H41122	Gamma-aminobutyric acid (GABA) A receptor, delta	3.59		2.49
AA434144	Claudin 3	3.51		
AA703187	Acetyl-coenzyme A transporter	3.50		2.27
H65328	RAB11B, member RAS oncogene family	3.48		0.43
H15456	Calpain, large polypeptide L1	3.46		
AA706301	Homeo box A5	3.45		
H03954	Human glucose transporter pseudogene	3.44		2.29
R53541	Chondroitin sulfate proteoglycan 4 (melanoma-associated)	3.42		
AA457298	Crystallin, gamma C	3.41		2.27
R36958	ESTs	3.38		2.02
AA182680	ESTs	3.38		
N35070	Tumor necrosis factor (ligand) superfamily, member 12	3.31	2.54	
AA485371	Bone marrow stromal cell antigen 2	3.27		
H02243	Homeo box B5	3.22	2.62	
AA001749	Microtubule-associated protein, RP/EB family, member 1	3.22		
AA186427	TRAF interacting protein	3.18		
R60317	Dihydrolipoamide dehydrogenase (E3 component of pyruvate dehydrogenase complex, 2-oxo-glutarate complex, branched chain keto acid dehydrogenase complex)	3.14		
AA133187	Iron-responsive element binding protein 2	3.10		
H15703	Myeloid/lymphoid or mixed-lineage leukemia 2	3.05		2.15
AA599145	ZW10 (<i>Drosophila</i>) homolog, centromere/kinetochore protein	3.03		2.62
AA039932	Thromboxane A2 receptor	3.02		
N30372	Interferon regulatory factor 5	3.02		
W69649	Mitogen-activated protein kinase kinase 5	3.01	2.42	2.27
AA102068	Heat shock transcription factor 4	2.70		
AA463452	DiGeorge syndrome critical region gene DGS1	-2.22	-2.56	-3.03
T49657	Potassium channel, subfamily K, member 3 (TASK)	-2.22	-3.03	
AA668527	Mucosal vascular addressin cell adhesion molecule 1	-2.33		
R51835	ESTs	-3.23		
N67778	Runt-related transcription factor 3		8.28	
AA481276	Autoantigen		2.51	
AA700054	Adipose differentiation-related protein; adipophilin		2.29	
R59927	Cytochrome c oxidase subunit VIc		2.21	
AA699361	Splicing factor 3b, subunit 4, 49 kDa		2.16	

Table 1 (Continued)

GenBank Accession #	Protein/gene	1 h	4 h	24 h
AA609982	DNA-binding transcriptional activator		2.12	
AA629567	Heat shock 70 kDa protein 10 (HSC71)		2.10	
N50854	Nucleolar protein 1 (120 kDa)		2.10	
R44334	Heat shock 90 kDa protein 1, beta		2.10	
N54794	Plasminogen activator inhibitor, type I		2.06	
T64223	Carboxypeptidase A3 (mast cell)		2.05	
N64628	Ubiquitin-like 4		2.02	
N92711	TATA box binding protein (TBP)-associated factor, RNA polymerase II, I, 28 kDa		2.00	
AA188761	Polymerase (DNA directed), gamma		-2.04	
AA157955	Sterol-C4-methyl oxidase-like		-2.08	-2.17
AA485214	Nucleobindin 2		-2.17	
AA157261	N-acetylglucosaminyl transferase component Gpi1		-2.33	
AA102634	TNF receptor-associated factor 5		-2.50	-2.56

Recent studies also suggest that HA may indirectly induce HSPs by acting on other kinases or phosphatases that then directly phosphorylate and activate HSF-1. It has been shown that activation of HSF-1 was possible even though it was not phosphorylated at any tyrosine residues.¹⁸ This finding implied that phosphorylation and de-phosphorylation take place at the tyrosine residue of a mediating kinase or phosphatase.¹³ In further support of this theory a study using genistein, a tyrosine kinase inhibitor employing a different mode of action, was unable to induce HSPs even when used at doses that inhibit tyrosine kinase activity.¹⁵ It has also been suggested that HA may play a role in the inhibition of I- κ B phosphorylation by preventing the activation and nuclear translocation of NF- κ B¹⁵. These findings therefore suggest a possible relationship between HA induction of HSPs and the inhibition of NF- κ B.

Gene expression profiling of HA treated cells suggested that cytoprotection from thermal insult might also be due to the regulation of transcription factors and inhibition of NF- κ B activation. While Western blotting confirmed the production of HSP40 and HSP70, the cDNA arrays exemplified the presence of mRNA for several HSPs in addition to several hundred additional genes. These results suggest that the effectiveness of HA in providing cytoprotection is likely attributable to the intricate relationship between HSP induction and the modulation of kinase activity and signal transduction.

Through gene expression profiling with cDNA arrays, we may better understand the benefits and targets of drug treatment. High-throughput screening with cDNA array technology may be applied to both drug discovery and development to determine the response of specific cell or tissue systems to a drug, or to evaluate a drug's mechanism of action. Currently, little is known about the cellular response to thermal injury from the therapeutic perspective, or how to rescue thermally injured tissue so that the effects of severe trauma and convalescence time can be reduced. In addition, since the wound healing response after thermal

injury is mediated by sub-lethally injured and surviving cells, the current findings may also have application in the development of treatments for small and blinding focal thermal injuries, such as those that occur after accidental laser exposure to the retinal macula.^{2,19} Furthermore, a better understanding of the HSP response may improve clinical applications of laser effects that are used in the treatment of photo-aged skin, corneal shape remodeling, cataract removal, transmyocardial revascularization, surgical excisions, hair removal, and pan-retinal photocoagulation in diabetic retinopathy. The determination that genes other than the HSPs are induced by HA suggests that alternate pathways may exist for therapeutic development of cytoprotectants.

MATERIALS AND METHODS

Cell Culture

ARPE-19 cells, derived from human retinal pigment epithelium (RPE), were a gift from Dr Larry Hjelmeland, Department of Ophthalmology, University of California at Davis. These cells exhibit a normal human diploid karyotype and an epithelial-like morphology.²⁰ Stock cultures were grown in T75 flasks in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 100 μ g/ml penicillin/streptomycin, and 5 μ g/ml fungizone (Life Technologies) at 37°C, 5% CO₂, and 95% relative humidity. During short periods of time when cells were used outside the incubator, they were placed in Modified Eagle Medium (MEM, Life Technologies) buffered with 20 mM HEPES, pH 7.4 (Sigma, St Louis, MO) and supplemented with 10% FBS, 100 μ g/ml penicillin/streptomycin, 5 μ g/ml fungizone (Life Technologies) to maintain pH.

HA Treatment

HA (Sigma, St Louis, MO) was reconstituted in 3% DMSO/DMEM to a stock concentration of 1 mg/ml and diluted to 0.10–1.00 μ g/ml with DMEM medium. For cell viability

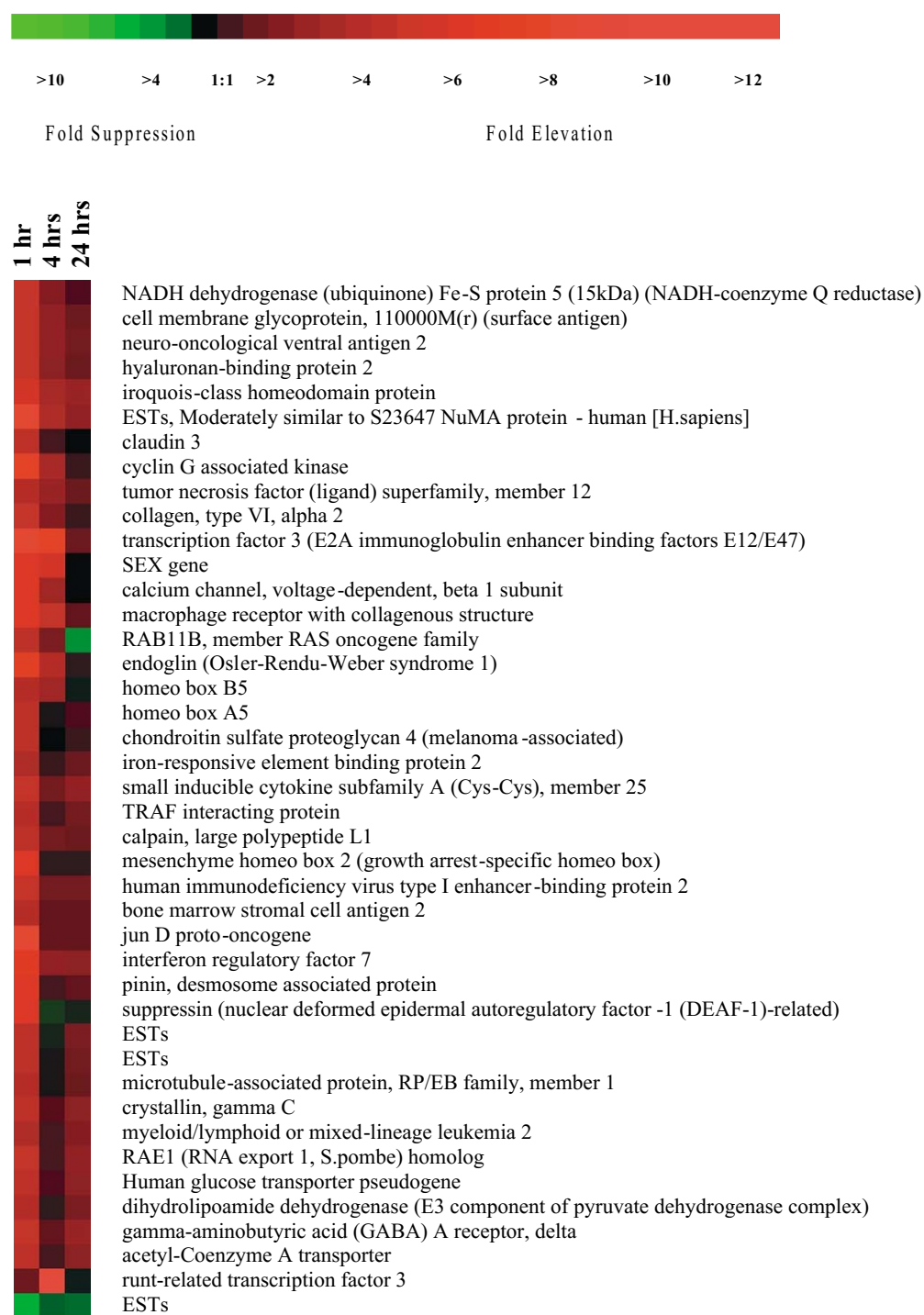


Figure 3 Gene clustering dendrogram of uninjured ARPE-19 cells treated with 1.00 µg/ml HA for 1,4, and 24 h. Genes undergoing ≥ 3-fold change in expression for at least one time point were clustered according to similarities in temporal expression patterns.

assessment, ARPE-19 cells in the experimental treatment groups undergoing thermal injury were treated with HA for 1 h, after which time the medium was replaced with regular DMEM, and the cells were allowed to respond for four additional hours before thermal injury.

For gene expression profiling with cDNA arrays, cells were seeded in 100-mm Petri dishes. At confluence, ARPE-19 cells were treated for up to 24 h with 1.00 µg/ml HA. Total RNA was isolated from cells after 1, 4, and 24 h of HA treatment. For Western blotting, protein was isolated from cells after 1,

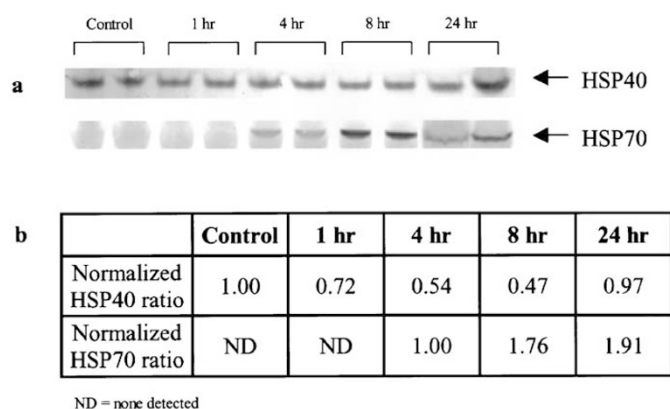


Figure 4 Time course for HSP production by uninjured ARPE-19 cells. Cells were treated with 1.00 $\mu\text{g/ml}$ HA for 1, 4, 8, and 24 h. (a) Translation of mRNA into protein was verified by Western blotting for heat shock proteins. (b) Normalized values (treated/untreated) for fold change in protein expression for HSP40 and HSP70.

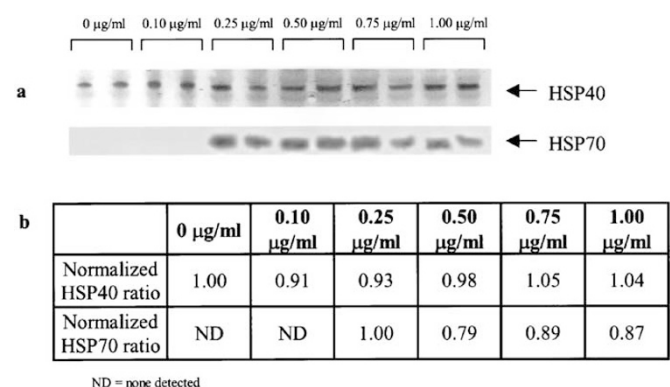


Figure 5 Dose-response determination for ARPE-19 cells by Western blotting. HSP proteins were assessed after 24 h of treatment with 0.10–1.00 $\mu\text{g/ml}$ HA. (a) Translation of mRNA into protein was verified for heat shock proteins. (b) Normalized values for fold change in protein expression for HSP40 and HSP70 (treated/untreated).

4, 8, and 24 h of treatment in 1.00 $\mu\text{g/ml}$ HA and after 24 h of treatment in 0.10–1.00 $\mu\text{g/ml}$ HA.

Thermal Injury Experiments

To determine the cytoprotective effectiveness of HA, progressively lethal thermal injuries were inflicted, resulting in a 40–50% decrease in cell viability at 24 h for untreated cells at the highest heating dose. ARPE-19 cells were seeded on 13-mm Thermanox coverslips (Nalge Nunc, Naperville, IL) in 24-well plates. Cells pretreated with HA for 1 h and placed in fresh medium without HA for 4 h were dipped in a 55°C heated HEPES saline bath for 6–8 s and then placed back into DMEM medium immediately after completion of all heating.

Cell Viability

Cell viability was assessed 24 h following thermal injury using Alamar Blue™ (Biosource International, Camarillo, CA), which is converted to a fluorescing compound in amounts proportional to the number of viable cells.²¹ The cells were incubated for 2 h at 37°C with culture medium containing 10% Alamar Blue™. After incubation, fluorescence was measured at 530 nm excitation and 590 nm emission (Cytofluor 2350, Millipore, Bedford, MA).

RNA Isolation

RNA isolation was performed following the Molecular Research Center Inc. (Cincinnati, OH) protocol for TRI Reagent®. RNA yield was determined spectrophotometrically and the quantity and quality of the RNA was estimated from absorption at 260 and 280 nm and gel electrophoresis on a 0.9% agarose gel containing 1:10 000 SYBR Gold nucleic acid stain (Molecular Probes, Eugene, OR). Only undegraded RNA free of genomic DNA contamination was used. Isolated RNA was solubilized in deionized H₂O and stored at –20°C until ³³P labeling.

cDNA Production

Two micrograms of total RNA were heated to 70°C for 5 min, then immediately frozen in liquid nitrogen and lyophilized. The lyophilized RNA was combined with 2 μg Oligo dT (1 $\mu\text{g}/\mu\text{l}$, Research Genetics, Huntsville, AL) and heated at 65°C for 5 min. A master mix containing 6 μl 5 × first strand buffer, 3 μl 0.1 M dTT (Life Technologies), 3 μl 5 mM dNTPs mixture, 1 μl RNase inhibitor (Eppendorf, Westbury, NY), 1.5 μl SuperScript II RT (200 units/ μl , Life Technologies), and 1 μl ³³P dATP (10 mCi/mmol, Amersham Pharmacia, Arlington Heights, IL) was then added and incubated at 39°C. The reaction was stopped by the addition of 4 μl EDTA (0.5 M, Ambion, Austin, TX). Hydrolysis of the RNA after reverse transcription was carried out at 68°C with 4 μl 1 M NaOH for 20 min. Unbound nucleotides were separated from the labeled probe by passage through a Centri-spin 20 column (Princeton Separations, Adelphi, NJ).

Hybridization and Phosphor Imaging

Blocking of nonspecific binding was performed in 5 ml Microhyb™ (Research Genetics) with 1.0 $\mu\text{g/ml}$ polydA (Research Genetics) and 1.0 $\mu\text{g/ml}$ Cot I DNA (GibcoBRL) at 42°C for 2 h. The purified probe was hybridized to an Atlas™ Human 1.2 cDNA Expression Array (Clontech, Palo Alto, CA) containing 1176 known genes at 42°C for 18–24 h in a 35 × 150 mm² roller bottle (Roller Oven, Hybaid Inc.). Washes were performed twice at 50°C in 20 ml 2 × SSC and 1% SDS for 20 min and once at 25°C in 100 ml 0.5 × SSC and 1% SDS for 15 min. The membranes were then placed on moistened Whatman filter paper and wrapped with plastic wrap before exposing to super resolution screens (Packard Instrument Co., Meridan, CT). Following exposure to the phosphor-imaging screens (1–4 days), the distribution and intensity of radioactivity were determined by scanning the screens with a phosphor-imager (Cyclone, Packard Instrument). The images were digitally acquired using OptiQuant

software (Version 3.0, Packard Instrument), and the autoradiographs were analyzed for changes in gene expression using Pathways software (version 3.0, Research Genetics).

Data Analysis

For analysis of gene expression, the ratios of the integrated optical densities for each cDNA target were compared between heated and control cells. Cluster analysis was then performed with Gene Cluster and TreeView software from Stanford University, courtesy of Dr Michael Eisen (<http://rana.lbl.gov/EisenSoftware.htm>).

Protein Isolation

Protein was extracted from the phenol phase remaining after extraction of RNA from the aqueous phase of cultured cells following the Molecular Research Center Inc. protocol for TRI Reagent[®]. The protein samples were solubilized in 500 µl of Lysis buffer (NOVEX, San Diego, CA) containing 1% β-mercaptoethanol (Sigma). When necessary, the samples were incubated at 50°C to effect solubilization.

Gel Electrophoresis, Total Protein Determination and Western Blotting

Ten microliters, corresponding to approximately 10 µg of protein from each treatment, were run on NuPage 4–12% Bis–Tris Gels (NOVEX) for 1 h at 125 mA. The protein was then transferred to a PVDF membrane (NOVEX) for 5 h at 95 mA (Semi-Dry Blotter II, Kem En Tec, Flanders, NJ). After transferring the protein, 2-methoxy-2,4-diphenyl-3(2H)-furanone (MDPF, Sigma) fluorescent stain was utilized for total protein quantification, following the procedure outlined by Alba and Dabar.²² MDPF fluorescently tagged all protein, and a relative total protein ratio was calculated by dividing the fluorescence value for each sample by that of the control. The ratio was then used to normalize for any variability in the amount of protein from sample to sample. The blots were hydrated in a 50% methanol/water solution for 10 min and then washed twice in 10 mM sodium borate, pH 9.5 for 5 min each time. After the second wash, the blots were incubated for 10 min in 10 ml borate buffer containing 50 µl (0.50%) 35 mM MDPF in DMSO. The blot was then rinsed briefly in borate buffer before visualizing under a UV light source. The total fluorescence for each sample was calculated using Gel-Pro Analyzer 2.0 software (Media Cybernetics, Silver Spring, MD).

Following total protein quantification, the membranes were hydrated in a 50% methanol/water solution for 10 min. The membranes were then incubated three times, 15 min each, in a blocking solution consisting of 0.2% I-Block (Tropix, Bedford, MA), 0.1% Tween-20 (Sigma) and 0.1% Thimerosal (Sigma) in PBS (Sigma). The blots were then incubated with a primary antibody for 2 h. Rabbit anti-human HSP40 (1:10 000 dilution) and mouse anti-human HSP70 (1:1000 dilution) were purchased from StressGen Biotechnologies Corp. (Victoria, BC, Canada). After the initial hybridization in primary antibody, the blots were washed another three times in blocking solution for 15 min

each. A 1:4000 dilution of alkaline-phosphatase labeled secondary antibody (goat anti-rabbit, Zymed, San Francisco, CA and goat anti-mouse, Tropix) was then added and the blots were incubated for another 2 h. Finally, the blots were washed three times in PBS, 15 min each, before staining. The blots were stained in a 10% NBT-BCIP solution (Zymed) until the bands developed. Quantitative analysis was performed using Gel Pro Analyzer 2.0 software on blots scanned into the computer.

Duality of Interest

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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