Multiple physical stresses induce γ-globin gene expression and fetal hemoglobin production in erythroid cells

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A B S T R A C T

Increased fetal hemoglobin (HbF) expression is beneficial for β-hemoglobinopathies patients; however, current inducing agents do not possess the ideal combination of efficacy, safety and ease of use. Better understanding the mechanisms involved in γ-globin gene induction is critical for designing improved therapies, as no complete mechanism for any inducing agent has been identified. Given the cytotoxic nature of most known inducing drugs, we hypothesized that γ-globin is a cell stress response gene, and that induction occurs via activation of cell stress signaling pathways. We tested this hypothesis by investigating the ability of physical stresses including heat-shock (HS), UV- and X-irradiation and osmotic shock to increase γ-globin gene expression in erythroid cells. Experiments in K562 and KU812 cells showed that each of these stresses increased steady-state γ-globin mRNA levels, but only after 3–5 days of treatments. HS and UV also increased γ-globin mRNA and HbF levels in differentiating primary human erythroid cells. Mechanistic studies showed that HS affects γ-globin mRNA at multiple levels, including nascent transcription and transcript stability, and that induction is dependent on neither the master regulator of the canonical HS response, HSF1, nor p38 MAPK. Inhibitor panel testing identified PI3K inhibitor LY294002 as a novel inducing agent and revealed potential roles for NFκB and VEGFR/PDGFR/Raf kinases in HS-mediated γ-globin gene induction. These findings suggest that cell stress signaling pathways play an important role in γ-globin gene induction and may provide novel targets for the pharmacologic induction of fetal hemoglobin.

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Introduction

Sickle cell disease (SCD) and β-thalassemia (β-thal) are human genetic diseases resulting in abnormal structure or decreased expression of the β-globin protein in red blood cells. These conditions represent a significant global health issue, with more than 330,000 affected infants born each year, 70% of whom are in Africa. Worldwide, 3.4% of deaths in children under five years old are attributed to these diseases and at least 5.2% of the population are carriers of a disease-causing β-globin gene variant [1]. In SCD, a single amino acid change in the β-globin protein leads to production of a sickle hemoglobin tetramer (HbS) that can polymerize and distort the shape and function of red cells [2,3]. β-Thal results from diminished or completely absent expression of the β-globin protein. SCD and β-thal patients suffer from many complications including anemia, hypertension, retinopathy and heart failure [4,5].

While allogeneic hematopoietic stem cell transplantation (HSCT) and gene transfer therapy have been applied to patients with SCD and β-thal, the modern medical facilities and financial resources required for these procedures are not available to most β-hemoglobinopathy patients [1]. A promising alternative therapeutic option is the pharmacologic induction of fetal hemoglobin (PIFH). Fetal hemoglobin (HbF) is comprised of two α-globin and two γ-globin chains (α2γ2) and is expressed at high levels during fetal and early postnatal periods. Its expression subsequently declines with a concurrent increase in adult hemoglobin (HbA, α2β2) expression. Increased HbF can lessen the severity of both SCD and β-thal [6–10]. PIFH also represents a potentially less expensive and more widely applicable means to treat β-hemoglobinopathies worldwide.

Over the last several decades, more than 70 pharmacologic agents have been shown to induce γ-globin expression and HbF production in various models. These agents include 5-azacytidine [9,11], decitabine [10], hydroxyurea [12,13] and butyrate derivatives [14], all of which are active in patients. These and other inducing agents are mechanistically diverse and include DNA methyl transferase inhibitors, histone deacetylase inhibitors, antimetabolites, mitotubule inhibitors, DNA damaging agents and immunomodulatory agents. The majority of these drugs are cytotoxic and/or alter global epigenetic and gene expression patterns, and thus are not ideal for long-term treatment of SCD and β-thal patients [15].
One feature common among many of these inducers is their ability to activate various cell stress signaling pathways including the mitogen-activated protein kinases (MAPKs), reactive oxygen species (ROS) and nitrogen oxide species (NOS) pathways [15]. Additionally, a few studies have suggested a direct role for the MAPKs p38 and ERK in the pharmacologic induction of γ-globin gene expression [16–18]. The cytotoxic nature of most HbF inducing agents, their documented ability to activate cell stress signaling pathways and lack of a mechanism to explain how diverse drugs induce γ-globin gene expression led us to propose the cell stress signaling model of HbF induction.

A key prediction of our hypothesis is that any stress, whether pharmacologic or physical, that activates cell stress signaling pathways should induce γ-globin gene expression and increase HbF levels. If physical stresses are able to induce γ-globin gene expression, this would support cell stress signaling as a common mechanism underlying γ-globin gene induction. If this model is correct, it would provide the first unifying theory to explain pharmacologic γ-globin gene induction. Many drugs targeting stress signaling pathways are currently being developed for a wide variety of medical conditions such as cancer and cardiovascular and neurodegenerative disorders. Therefore, verifying that these pathways are involved in HbF induction could lead to the rapid adoption of new targeted therapies for SCD and β-thal.

To test our hypothesis, we applied heat shock (HS), ultraviolet (UV)- and X-irradiation and osmotic shock, all well-defined activators of cell stress signaling, to K562 and KU812 erythroleukemia cell lines and primary human erythroid cells to determine if they could activate γ-globin gene expression. We also used these physical stressors to more closely examine the role of cell stress signaling in γ-globin gene and HbF induction to further our mechanistic understanding of current inducing agents, as well as to discover targets that might be pharmacologically modulated to induce HbF in patients.

Materials and methods

Cells, plasmids and reagents

K562 cells were maintained in RPMI 1640 with l-glutamine (Cellgro, Manassas, VA) supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO2. Granulocyte-colony stimulating factor-primed peripheral blood CD3+ cells were obtained from the hematopoietic cell-processing core at the University of Washington or from Dr. Patrick Gallagher of Yale Medical School using institutional review board-approved protocols. Cultures were maintained as described in Sankaran et al. [19]. Briefly, cells were expanded for seven days in medium containing FLT-3 ligand, SCF, IL-3 and IL-6. On day seven, cytokerines were changed to EPO, IL-3, SCF and β-estradiol for erythroid differentiation through day 20. pCDNA3-Flag MMK6 (glu) was purchased from Addgene (Addgene plasmid 13518) [20]. JNK inhibitor VIII (Calbiochem), Sorafenib (Selleck Chemicals, Houston, TX), Actinomycin D (ActD), U0126, Q-VD-OPh hydrate, LY294002 and BMS-345541 (all purchased from Sigma-Aldrich, St. Louis, MO), were dissolved in dimethylsulfoxide and stored at –20 °C.

Physical stress treatments

K562 and KU812 cells were suspended at 3.0 × 10⁵ cells/mL in RPMI media. For heat shock (HS), T25 tissue culture flasks (Thermo Fisher Scientific, Chicago, IL) containing 10 mL of media were immersed in a water bath at the indicated temperature for 10–90 min daily. HS treatments were performed beginning 24 h after siRNA or plasmid transfections or one hour after drug treatment. For UV irradiation, cells were transferred to 10 cm² tissue culture plates at a cell density of 3.0 × 10⁵ cells/mL and exposed to 30–65 μJ of UV light daily using a UV Stratalinker 1800 (Stratagene, La Jolla, CA) according to manufacturer’s instructions. For X-irradiation, cells in T25 flasks were exposed to 0.25–1.5 Gy from a Cs-137 irradiator daily. For osmotic shock, cells were resuspended in media at the indicated osmolarity for 10 min and then returned to normal osmolarity media (290 mOsm). Hypersmotic media was made by increasing concentrations of NaCl. Hypersmotic media was made by water dilution. Primary erythroid progenitor cells were treated as above with HS, UV or X-irradiation at the doses and schedules as indicated from day 9 through 17 of differentiation.

Real-time quantitative reverse-transcription PCR and hemoglobin analysis

Total RNA was isolated from cells using RNeasy columns (Qiagen, Germantown, MD) according to manufacturer’s instructions. cDNA was generated from equal amounts of RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) and quantitative polymerase chain reaction (qPCR) was performed with iq SYBR Green Supermix (Bio-Rad). Gene expression levels were calculated by the method of Livak et al. [21], relative to GAPDH or RPL13A gene expression. PCR primers are listed in Table 1.

Hemoglobin analysis was performed on lysates from primary erythroid cells at day 20 of in vitro differentiation with 2 × 10⁵ cells per sample. Hemoglobin content was calculated from the absorbance at 415 nm using the extinction coefficient of 125 mM⁻¹ cm⁻¹ [22]. Hemoglobin high-performance liquid chromatography (HPLC) was performed as described in Ou and Rongnerud [23] using a PolyCAT-Acation exchange column (The Nest Group, Southborough, MA).

Western blot analysis

Protein lysates were prepared in equal parts Bradford Lysis Buffer (25 mM HEPES pH 8.0, 1% v/v Triton X-100, 10% v/v glycerol) and Laemmli buffer (Bio-Rad) supplemented with 100 mM DTT, complete, Mini, EDTA-free Protease Inhibitor Tablets (Roche Diagnostics, Indianapolis, IN) and phosphatase inhibitors (Sigma-Aldrich P5726, P0044). Protein was quantified by RED660 Protein Assay (G-Biosciences, St. Louis, MO) according to manufacturer’s instructions. Equal micrograms of protein were electrophoresed and transferred to Immobilon-P membranes (Millipore, Billerica, MA). Membranes were incubated overnight at 4 °C with the following antibodies: p-p38 (1:1000, Cell Signaling Technology 9211), total p38 (1:1000 Cell Signaling Technology 9212), FLAG (1:1000, Sigma F7415) and β-actin (1:30,000, Sigma A1978). siRNA and plasmid transfections

K562 cells were transfected with 100 nM p38 siRNA (Santa Cruz Biotechnology, sc-29433), 50 nM HSFl siRNA (Ambion, Life Technologies, 1156740) or Silencer® negative control No. 1 siRNA (Ambion, Life Technologies, AM4611) for 24–96 h. HiPerfect transfection reagent (Qiagen) was used according to manufacturer’s instructions. Plasmid transfections were performed using 1.6 μg of pCDNA3.1 vector or pCDNA3-Flag MMK6 (glu) and Lipofectamine 2000 reagent (Invitrogen, Life Technologies) according to manufacturer’s instructions.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Quantitative real-time RT-PCR primers.</th>
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<tr>
<td>Gene</td>
<td>Forward (5′–3′)</td>
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<tr>
<td>c-Jun</td>
<td>ATCGACATGAGCTGCCAGGA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TATCCATCACTCTTCCA</td>
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<tr>
<td>HSFl1</td>
<td>GCTACAAGATCCTGACAAGA</td>
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<tr>
<td>HSFl27</td>
<td>TACAGCCGCACTGGCGCCAC</td>
</tr>
<tr>
<td>Nascent γ-globin</td>
<td>TATGCGCTTCGGTGACCGACA</td>
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<tr>
<td>p38</td>
<td>GTGCCCTCCGGTGACCGACA</td>
</tr>
<tr>
<td>RPL13A</td>
<td>CCTGGAAGCACAAGAAGAAGA</td>
</tr>
<tr>
<td>β-Globin</td>
<td>AGATCTGCTGGCCACGCC</td>
</tr>
<tr>
<td>γ-Globin</td>
<td>AGACGCCATCGGTCATACCCCA</td>
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Reverse (5′–3′) |  
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<tr>
<td>CACCTTCTCCGGTACATTG</td>
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<td>CATGCCACCGATCTTCC</td>
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<td>CTGACAGGTTGACGCAAGA</td>
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<tr>
<td>TACATCTTCGGTGACCGACA</td>
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<tr>
<td>GTGACAGGTTGCCGACCGAC</td>
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<td>TCTGACTGGTCCGACCC</td>
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Statistical analysis

P-values were determined using a one-way ANOVA with a Tukey’s multiple comparison test for Figs. 1 and 2, and unpaired student’s t test for Figs. 5 and 6 using GraphPad Prism. Data are presented as ±SEM unless otherwise noted.

Results

Heat shock induces γ-globin gene expression in K562 cells

Our model is based on the hypothesis that cell stress signaling plays a central role in the pharmacologic induction of γ-globin gene expression. If this hypothesis is correct, then any stress that activates relevant cell stress signaling pathways, whether chemical or physical, should also increase γ-globin gene expression. To test our model, we first examined the ability of heat shock (HS) to induce γ-globin gene expression in the K562 erythroid cell line. This cell line has been used for many years to study γ-globin induction by drugs. Many agents that activate the γ-globin gene in K562 cells also activate γ-globin gene expression and HbF production in primary human erythroid cells and/or in human subjects [15].

Preliminary experiments showed that HS for 45 min at 43.5 °C on consecutive days produced a 1.7-fold increase in γ-globin mRNA when measured 24 h after the first HS and a 2.6-fold response 24 h after the second HS (Fig. 1A). Increasing the number of HSs to 5 consecutive days did not further augment γ-globin induction (data not shown). We then evaluated how variables including temperature and

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**Fig. 1. Heat shock induces γ-globin gene expression in erythroleukemia cell lines. HS was performed daily for three consecutive days. γ-Globin mRNA was measured daily by RT-PCR. (A) Effect of HS on steady-state γ-globin mRNA levels. K562 cells were exposed to HS at 43.5 °C for 45 min. (B) Effect of HS temperature on γ-globin mRNA levels. K562 cells were exposed to HS for 45 min. at 37 °C or 43 °C. (C) Effect of HS length on γ-globin mRNA levels. K562 cells were exposed to HS at 43.5 °C for 0, 10, 15, 30, 45, 60 or 90 min. (D) Effect of HS length on K562 cell growth. (E) Effect of HS on steady-state γ-globin mRNA levels in KUB12 cells. Error bars indicate ± 1 SEM (A and B) or ± 1 std dev (B and C). Data in A and D are from 5 and 2 independent experiments, respectively. Data in B and C are from a representative experiment with 3 replicates of each data point. ** indicates P < 0.0001.**
duration of HS affect γ-globin mRNA induction. We found that the magnitude of the response was sensitive to small changes in temperature, with 43 °C causing a 2.6-fold induction and 43.5 °C causing a maximal 3.5-fold increase in γ-globin expression compared to the 37 °C control (Fig. 1B). Heat shocking at 42 °C or 44.5 °C did not increase γ-globin RNA, while the higher temperature drastically slowed cell growth (data not shown). To determine the effect of HS duration on γ-globin induction, K562 cells were subjected to two consecutive days of 43.5 °C HS, with exposures ranging from 10 to 90 min. As shown in Fig. 1C, there was a clear correlation between increased HS length and γ-globin expression with increased exposure time up to 45 min, to a maximal induction of 2.4-fold compared to the unstressed control. Fig. 1D shows that HSs lasting 45 min or longer decreased cell proliferation, suggesting that we were delivering doses that activate cell stress responses including growth suppression. We subsequently used daily 45 min HSs at 43.5 °C as our standard condition.

To determine whether the ability of HS to induce γ-globin gene expression was limited to K562 cells, we applied our optimized HS conditions to another erythroleukemia cell line, KU812. As shown in Fig. 1E, HS of these cells produced a similar induction of γ-globin mRNA. These results indicate that HS can effectively induce γ-globin expression in two independent erythroid cell lines in a temperature- and time-dependent manner.

UV light, X-irradiation and osmotic shock also induce γ-globin gene expression

To determine if the induction of γ-globin mRNA was unique to HS, we next tested other physical stresses. K562 cells were treated daily with 30, 40, 50, 60 or 65 μJ of UV irradiation. Unlike HS, UV did not significantly increase γ-globin gene expression until day 5, peaking at
2.64-fold over control for the 65 μJ dose (Fig. 2A). This result was recapitulated in the KU812 cell line using a 60 μJ dose (Fig. 2B).

We next examined the effect of X-irradiation on γ-globin expression by subjecting K562 cells to increasing doses ranging from 0.5 to 1.5 Gy daily for 5 days. We observed a significant dose-dependent induction of γ-globin mRNA on days 4 and 5, reaching a maximum of 2.0-fold induction with 1.5 Gy on day 5 (Fig. 2C). Similar induction was also seen with the KU812 cell line (Fig. 2D). Finally, we tested the effects of osmotic shock in K562 cells and found that hyper-, but not hypo-, osmotic shock also showed an ability to induce γ-globin mRNA after 5 days (Fig. 2E).

Our results with HS, UV- and X-irradiation and osmotic shock demonstrated that four distinct physical activators of cell stress signaling can induce γ-globin expression in erythropoiesis cell lines. While these results are consistent with our model, stress activation of γ-globin gene expression appears to be a complex process, requiring multiple stress applications over multiple days.

Physical stresses induce γ-globin gene expression in differentiating human primary erythroid progenitors

To test the ability of physical stresses to induce γ-globin gene expression in human primary cells, we used a previously described 2-phase in vitro erythroid differentiation system in which G-CSF-mobilized CD34+ peripheral blood cells differentiate into mature erythroid precursor cells over 20 days [19]. In a single experiment for each stress, we applied HS, UV and X-ray stresses from day 9 through 17 of differentiation. This schedule was based on previous published data showing that daily application of 5-azacytidine over this time period was optimal for γ-globin induction in this system [24]. For HS, cells were exposed for 45 min to 41.5 °C every day or 43.3 °C every other day. In UV experiments, cells were treated with 50 μJ every day or 60 μJ every other day. For HS and UV, γ-globin and β-globin mRNA levels were monitored throughout differentiation and reported as the fold change of the area under the curve (AUC) of the γ/γ + β ratio. This provides a measure of the total amount of each mRNA available for translation throughout differentiation.

As seen in Fig. 3A, HS increased the γ/(γ + β) ratio of globin mRNA 1.24-fold over baseline at 41.5 °C and 1.85-fold at 43.3 °C. The increased percentage of γ-globin transcript was accompanied by a corresponding increase in the percentage of fetal hemoglobin (HbF) from 2.7% to 4.5% and 4.8% (1.7- and 1.8-fold respectively, Fig. 3B). With both UV treatments, total globin transcripts and hemoglobin content were reduced. However, the γ/(γ + β) ratio was increased by 1.43-fold with the 50 μJ treatment (Fig. 3C). Interestingly, UV had a greater effect on increasing the percentage of HbF compared to the mRNA ratio in erythroid progenitors, with both 50 and 60 μJ treatments resulting in a 2.1-fold increase over baseline (2.8% to 6.0%, Fig. 3B). Representative HPLC curves are shown in Fig. 3D.

For X-ray experiments, cells were treated with 0.25, 0.5 or 1.0 Gy daily and γ-globin mRNA was measured only on day 11 of differentiation. Doses of 0.25 and 0.5 Gy resulted in 4.1 and 4.3-fold increases in γ-globin mRNA on day 11 of differentiation compared to untreated control (Fig. 3E). Response to the 1.0 Gy dose was less and inhibition of cell growth and differentiation were noted. These results confirm that physical stresses induce γ-globin gene expression and also induce HbF in differentiating primary human erythroid cells.

Heat-shock affects γ-globin mRNA levels by multiple mechanisms

The mechanisms involved in stress-induced γ-globin gene expression appear complex, requiring multiple applications of each stress over 3–5 days to achieve maximal expression. The above results demonstrate that unlike canonical early stress-response genes such as those in the HS protein (Hsp) family [25], and immediate early genes like c-jun and c-fos [26], γ-globin gene expression does not respond rapidly to physical stress. To ensure that typical early response genes were responding as expected in K562 cells, we followed changes in c-jun mRNA after HS. The contrast between c-jun and γ-globin induction in K562 cells is apparent in response to three daily HS treatments (Fig. 4A). c-jun expression is rapidly induced 6.8-, 4.2- and 3.4-fold after the first, second and third HSs and returns to baseline levels between HSs. In contrast, γ-globin expression did not reach maximum induction of 2.3-fold until the third HS.

To further investigate the changes in γ-globin steady-state mRNA levels in response to stress we determined the effect of HS on nascent γ-globin transcript levels using PCR primers that crossed an intron-exon boundary. We subjected K562 cells to 43.5 °C HS for 45 min daily for three days, and then quantified nascent transcript levels and γ-globin steady-state mRNA before and after each HS. As shown in Fig. 4B, nascent transcript levels followed a cyclic pattern. While initially suppressed, transcript levels rose higher than baseline after 24 h post-HS. This initial decline was seen again after the second HS, followed by an amplified increase compared to the first HS. The first and second HS had a 1.6- and 2.6-fold increase over unstimulated control respectively. Steady-state γ-globin mRNA levels also initially declined after each HS but then gradually increased over the same time period, to reach a maximal 2.7-fold induction after the third HS (Fig. 4C). Comparison of nascent versus steady-state γ-globin mRNA responses suggests that the increase in nascent transcription following each stress application contributes to the overall accumulation of steady-state γ-globin mRNA.

Another possible contributing factor in increased steady-state mRNA levels is enhanced transcript stability. To examine this, K562 cells were exposed to HS for 45 min at 43.5 °C for two consecutive days. We then applied the transcriptional inhibitor Actinomycin D (ActD) to control and stressed cells immediately after the second heat-shock to halt transcription and then monitored γ-globin transcript levels for 12 h. In contrast to our expectations that HS might increase γ-globin mRNA stability, we found that the treatments accelerated γ-globin transcript degradation compared to control cells (Fig. 4D). These results indicate that HS produces a complex set of effects on γ-globin mRNA. These include suppression of transcription in the first few hours after the stress is applied, followed by a longer period of increased transcription that is accompanied by a shortening of the mRNA half-life. The end result of these effects is a relatively slow increase in γ-globin mRNA following HS.

Heat-Shock Transcription Factor 1 (HSF1) is not required for γ-globin induction

The above findings demonstrate that HS clearly affects the transcriptional activation of the γ-globin gene; therefore, we looked at the involvement of HSF1, the master transcriptional regulator of the canonical HS response. HSF1 is a helix–turn–helix transcription factor that coordinates the rapid up-regulation of many genes including those coding for heat shock proteins (Hsp) and chaperones [27]. HSF1 is also involved in responses to other physical and chemical insults including oxidative stress, heavy metals, viral infection and chemical agents [28–30]. To determine whether HSF1 plays a role in γ-globin gene induction by HS, we performed siRNA knockdown of HSF1 in K562 cells during our standard HS protocol of 43.5 °C for 45 min daily for three days. As shown in Fig. 5A, HSF1 mRNA levels were suppressed throughout the treatments, reaching 73% knockdown at 48 h compared to cells treated with control siRNA. This level of HSF1 knockdown was sufficient to completely suppress Hsp27 induction following HS (Fig. 5B). Hsp27 is a well-described transcriptional target of HSF1, and is activated both transcriptionally and post-transcriptionally in response to cell stresses including HS [28]. Despite this effective inhibition of the HSF1–mediated post-HS transcriptional response, HS induction of γ-globin gene expression was unaffected by HSF1 knockdown, suggesting that this response is distinct from the canonical transcriptional stress response to HS in K562 cells (Fig. 5C).
Genetic manipulation of p38 MAPK does not modulate γ-globin gene expression

Given that HSF1 does not appear to regulate HS induction of γ-globin, we hypothesized that the p38 MAPK signaling pathway could be the key mediator of stress induction of γ-globin mRNA. p38 MAPK is a cell stress signaling kinase that plays a major role in cellular responses to physical stresses including HS, UV and X-irradiation and osmotic shock [31–33]. p38 has also been implicated in the induction of γ-globin gene expression by several drugs [16–18,34]. To determine whether p38 was required for HS-mediated γ-globin induction, we used siRNA to reduce p38 mRNA and protein levels throughout our usual 3 day treatment period (Figs. 6A and B). Despite this reduction in both total and phospho-p38, γ-globin mRNA induction by HS was just as effective as in untransfected and control siRNA samples (Fig. 6C). While this result suggests that HS-induced γ-globin expression is independent of p38, it is possible that a small amount of residual p38 was able to activate the γ-globin gene response. To further characterize the HS response, we next investigated the role of direct p38 activation, via its major upstream activator MKK6, in γ-globin induction. We transfected a flag-tagged, constitutively active MKK6 construct (pcDNA3-Flag MKK6(Glu)) [20] into K562 cells for 24, 72 and 96 h. Western blotting showed expression of MKK6-Glu and increased phospho-p38 compared to un-transfected or vector controls at each of these time points (Fig. 6D). Despite increased p38 activation, γ-globin mRNA expression was unchanged from un-transfected or vector control.
cells at each time point (Fig. 6E). Collectively, these results suggest that p38 activation is neither necessary nor sufficient to induce γ-globin expression, and is therefore not a key component of the γ-globin physical stress response.

Inhibition of major cell signaling pathways have varied effects on HS-mediated γ-globin gene induction

Because common stress pathways involving HSF1 and p38 MAPK did not appear to mediate stress-induced γ-globin expression, we next investigated the involvement of several other signaling pathways in γ-globin mRNA induction by HS. Using select inhibitors, we examined the role of caspase, JNK, ERK, PI3K, NFκB, and VEGFR/PDGFR/Raf kinase signaling in HS-mediated γ-globin gene induction.

We chose to examine each of these pathways because of their distinct connections to the cellular stress response. Caspases can be activated by a variety of stresses, and caspase-3 has been implicated in the erythroid differentiation process [35,36]. Both JNK and ERK are MAPKs that respond to mitogenic stimuli and stresses including HS, UV- and X-irradiation [31]. Similar to ERK, the PI3K pathway is most notably involved in cell growth and proliferation. Activated PI3K signals through AKT/mTOR in response to various stimuli including growth factors, energy status and cellular stress [37]. The NFκB pathway also acts as a primary regulator of the stress response, enacting protection against many exogenous or endogenous agents by regulating a diverse set of genes [38]. Finally, Raf kinases mediate MEK/ERK signaling and Raf inhibitors have been shown to activate ERK under wild-type Raf conditions [39].

Implicating one of these pathways in HS-mediated γ-globin induction would provide valuable mechanistic insight.

First, we subjected K562 cells to the standard HS treatment in the presence of 10 μM of the pan-caspase inhibitor Q-VD-OPh (QVD). This dose inhibits caspases 3, 7 and 8 and prevents cleavage of PARP-1 [40]. As shown in Fig. 7A, caspase inhibition did not affect γ-globin induction. Similar results were obtained with 10 μM of the JNK inhibitor VIII (EC₅₀ = 920 nM) [41], implying that both caspases and JNK are not mediating HS induction of γ-globin mRNA (Fig. 7B).

We next determined if ERK signaling was important in HS-mediated γ-globin induction using the MEK inhibitor U0126. U0126 has been shown to induce γ-globin expression in K562 cells, although the mechanism of this effect is not known [18,42]. Consistent with those reports, 10 μM U0126 increased γ-globin mRNA levels 6.5-fold compared to unshocked K562 cells. HS and U0126 treatments additively induced γ-globin expression, resulting in 2.0- and 6.5-fold increases alone, and an 8.4-fold increase when in combination (Fig. 7C). These results suggest that HS-mediated γ-globin induction is independent of ERK signaling.

Finally, we tested the effects of NFκB inhibitor BMS-345541 and tyrosine protein kinase and Raf kinase inhibitor sorafenib. In contrast to the previously tested inhibitors, both 5 μM BMS-34551 and 10 μM sorafenib treatments abolished γ-globin induction when combined with...
HS (Figs. 7E and F). These results suggest a potential mechanistic role for the NFκB pathway and tyrosine protein or Raf kinases in HS-mediated γ-globin induction.

Discussion

The idea that pharmacologic reactivation of the fetal β-like globin genes in people with β-hemoglobinopathies could produce a significant therapeutic benefit was proposed more than 30 years ago [6]. Since that time, multiple studies have verified this concept by demonstrating benefits to patients [9,11,12,14] and by discovery of more than 70 compounds that activate γ-globin gene expression in a variety of systems. Despite this progress, hydroxyurea (HU) remains the only FDA-approved drug that provides HbF induction and HU has several shortcomings, including myelosuppression and a lack of efficacy in many SCD and β-thal patients [43]. Other inducing agents generally have excessive side effects that have made them unacceptable for long-term use in hemoglobinopathy patients. Central to designing improved agents for HbF induction in patients is an understanding of the mechanisms by which γ-globin gene expression is activated. To date, no complete mechanism has been verified for any inducing agent. The fact that most known inducing agents are cytotoxic chemotherapeutic drugs led us to propose a unified model to explain the ability of this group of mechanistically distinct compounds to induce γ-globin gene expression [15]. This model was based on the hypothesis that most inducing agents increase γ-globin gene expression through activation of cell stress signaling pathways. A key prediction of this model is that any stress, including physical stresses, should also activate γ-globin gene expression. Our experiments have confirmed this prediction for HS, UV- and X-irradiation and osmotic shock in two erythroleukemia cell lines and human primary erythroid cells.

Another reason for examining the effects of physical stresses on γ-globin gene expression is that the cellular and gene expression effects of physical stresses have been studied in a wide variety of experimental systems over many years. It was our hope that these prior studies would provide insights into how physical stresses and drugs induce γ-globin gene expression. Unexpectedly, our experiments show that, in contrast to the typical rapid up-regulation of stress-induced gene expression seen for most stress response genes, nascent γ-globin gene expression is initially suppressed for several hours before it increases. Depending on the stress used, this response takes 3–5 days to reach a peak steady-state mRNA response. In addition, HS experiments show that in contrast to the overall slow rise in γ-globin mRNA, there is a decline in mRNA stability following HS. We also found that neither HSF1, the key transcriptional regulator of a wide array of stress response genes, nor p38 MAPK cell stress signaling pathway appear to be involved in γ-globin gene induction by HS. Several publications have suggested that some γ-globin inducing drugs such as sodium butyrate [16], hydroxyurea [34] and valproate [18] work through this pathway. However, we found that knock-down of p38 and P-p38 using siRNA does not inhibit HS-mediated γ-globin mRNA induction. While it is possible that there was sufficient residual P-p38 to activate γ-globin expression despite the knock-down, we also found that direct phosphorylation of p38 by a constitutively active MKK6 protein did not increase γ-globin gene expression. Taken together, these experiments indicate that γ-globin gene induction by physical stress is not accomplished through common stress pathways involving HSF1 or p38 MAPK.

After ruling out involvement of HSF1 and p38 MAPK, we next determined the effects of pharmacologic inhibitors targeting other major cell signaling pathways including ERK, JNK, PI3K, Nf-κB, VEGFR/PDGFR/Raf kinases and the caspases. We found that both JNK and caspase inhibition had no effect on basal or HS-induced γ-globin expression. Similarly,
while ERK inhibition alone was an effective inducer of γ-globin mRNA, inhibition of the pathway did not alter the HS response.

A surprising result from inhibitor panel testing was that, like U0126, the PI3K inhibitor LY294002 increased γ-globin mRNA levels in both unshocked and shocked cells. This effect has not been previously reported and may represent an intriguing target for γ-globin induction. Reaching as high as a 16-fold increase, LY294002 induces γ-globin gene expression more than any other treatment we have observed, and is much more than the 2.0-fold that is typically seen with HU in K562 cells, for example [44]. Currently, drugs targeting the PI3K pathway are under development for cancer and other diseases [45] and may thus be an exciting new avenue to explore for γ-globin gene induction. Despite this finding, PI3K inhibition does not alter the HS response, suggesting it is not a part of the physical stress mechanism.

In contrast, both NFκB inhibition by BMS-345541 and tyrosine protein kinase and Raf kinase inhibition by sorafenib abolished HS induction of γ-globin mRNA. These results provide valuable mechanistic insight into the HS process and suggest they are important pathways to explore in relation to physical stress-mediated γ-globin induction. The sorafenib result is notable in its ability to inhibit γ-globin expression. However, its broad-spectrum inhibitory profile of tyrosine protein and Raf kinases implicate any one of a number of complex pathways. In contrast, the NFκB pathway represents a more specific and intriguing potential component of the HS response as it is known to be activated.

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**Fig. 6.** Genetic manipulation of p38 MAPK does not modulate γ-globin gene expression. (A–C) K562 cells were transiently transfected with siRNA specific for p38. 24 h later, cells were heat-shocked at 43.5°C for 45 min. for three consecutive days; RNA was collected each day and protein was collected on day 3. No siRNA and control siRNA (siCtl) were used as controls. (A) qPCR results measuring p38 steady-state mRNA levels throughout treatment. (B) Phospho- and total p38 protein levels in unshocked and post-HS cells on day 3. (C) Effect of HSF1 siRNA on γ-globin mRNA levels after third HS. Real-time results expressed as fold over unshocked siCtl. Error bars denote ± SEM, n = 2. (D) and (E) K562 cells were transiently transfected with pcDNA3-Flag MKK6(glu) and protein and RNA samples were collected at 24, 72 and 96 h post-transfection. No transfection (UT) and pcDNA3.1 vector were used as controls. (D) pcDNA3-Flag MKK6(glu) and phospho-p38 protein levels following transfection. (E) Effect of pcDNA3-Flag MKK6(glu) on γ-globin mRNA levels. Results expressed as fold over UT. Error bars denote ± 1 stdev.
by physical stresses and chemotherapeutic agents that induce γ-globin expression. NF-κB is involved in both activation and suppression of various erythroid genes throughout differentiation including α-globins and NF-E2 [46]. Consequently, while its role in HS-mediated γ-globin gene induction needs to be more fully explored, NF-κB appears to be an important mechanistic contributor to the process.

Our experiments have demonstrated a role for cell stress signaling in γ-globin gene induction and suggest that this induction is part of a physiologically important stress response in erythroid cells. Recent work from our lab has also implicated the NRF2 antioxidant response and integrated stress response pathways in increased HbF production [47–49]. How increased levels of HbF might be protective against stress remains to be determined, but there is evidence that other globin proteins including neuroglobin [50], cytoglobin [51] and even microbial globins [52] can protect against oxidative and nitrosative stresses. Our findings suggest that cell stress signaling pathways not only offer potential targets for the pharmacologic induction of fetal hemoglobin, but also may serve an important role in red cell physiology.

Conflict of interest

The authors declare no competing financial interests.

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