Original Research *Feature article*

δ-Aminolevulinate induces fetal hemoglobin expression by enhancing cellular heme biosynthesis

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Impact statement

Inherited mutations in the β-globin-like genes result in the most common forms of genetic blood disease including sickle cell disease (SCD) and β-thalassemia worldwide. Therefore, effective inexpensive therapies that can be distributed widely are highly desirable. Currently, drug-mediated fetal hemoglobin (HbF) induction can ameliorate clinical symptoms of SCD and β-thalassemia and is the most effective strategy for developing new therapeutic options. In the current study, we confirmed that δ-Aminolevulinate (ALA), the precursor of heme, induces γ-globin expression at both the transcriptional and translational levels in primary human erythroid progenitors. Moreover, the results indicate activation of the transcription factor NRF2 (nuclear factor (erythroid-derived 2)-like 2) by ALA to enhance HbF expression. These data support future study to explore the potential of stimulating intracellular heme biosynthesis by ALA or similar compounds as a novel therapeutic strategy for treating SCD and β-thalassemia.

Abstract

Sickle cell disease (SCD) and β-thalassemia are inherited blood disorders caused by genetic defects in the β-globin gene on chromosome 11, producing severe disease in people worldwide. Induction of fetal hemoglobin consisting of two α-globin and two γ-globin chains ameliorates the clinical symptoms of both disorders. In the present study, we investigated the ability of δ-aminolevulinate (ALA), the heme precursor, to activate γ-globin gene expression as well as its effects on cellular functions in erythroid cell systems. We demonstrated that ALA induced γ-globin expression at both the transcriptional and protein levels in the KU812 erythroid cell line. Using inhibitors targeting two enzymes in the heme biosynthesis pathway, we showed that cellular heme biosynthesis was involved in ALA-mediated γ-globin activation. Moreover, the transcription factor NRF2 (nuclear factor [erythroid-derived 2]-like 2), a critical regulator of the cellular antioxidant response, was activated by ALA and contributed to mechanisms of γ-globin activation; ALA did not affect cell proliferation and was not toxic to cells. Subsequent studies demonstrated ALA-induced γ-globin activation in erythroid progenitors generated from normal human $CD34^+$ stem cells. These data support future study to explore the potential of stimulating intracellular heme biosynthesis by ALA or similar compounds as a novel therapeutic strategy for treating SCD and β-thalassemia.

Keywords: δ-Aminolevulinate, fetal hemoglobin, heme biosynthesis, reactive oxygen species, NRF2, globin expression

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Introduction

Inherited mutations in the β-like globin genes cause the most common forms of genetic blood disorders, the β-hemoglobinopathies worldwide. Some naturally occurring mutations improve the clinical symptoms of sickle cell disease (SCD) and β-thalassemia through producing hereditary persistence of fetal hemoglobin (HbF) expression. This observation provides the rationale for studies to define mechanisms of drug-mediated HbF induction as an effective strategy for treating β-hemoglobinopathies. Hydroxyurea (HU) is the only Food and Drug Administration-approved HbF-inducing agent.[1] The molecular mechanism of HbF induction by HU involves the generation of nitric oxide and activation of the cyclic guanosine monophosphate signaling pathway.[2] Currently, the most promising new HbF-inducing agent is the DNA methyl transferase inhibitor, decitabine. The recently completed Phase II trial demonstrated decitabine combined

with tetrahydrouridine effectively blocked its metabolism after oral administration to enhance HbF expression in SCD patients[3] through γ-globin promoter hypomethylation, thus allowing transactivation by DNA-binding protein.[4,5]

The production of hemoglobin requires coordinated synthesis of the globin chains and heme; however, excess unbound molecules are toxic to cells. Heme is ubiquitously produced in cells as a prosthetic group of many hemoproteins for diverse functions including the regulation of globin chain synthesis.[6–8] There are eight enzymes involved in catalyzed reactions for heme synthesis across two subcellular compartments: the mitochondrion and cytoplasm.[9] Among them, δ-aminolevulinate synthase (ALAS) catalyzes the first and the rate limiting reaction to produce ALA. Thus, exogenously added ALA can bypass the ALAS-mediated reaction and accelerate heme production,[10] as well as enhance globin messenger RNA (mRNA) translation and hemoglobin synthesis in cell culture systems.[6,11] Although ALA is critical for heme biosynthesis, its role in molecular mechanisms of globin genes regulation has not been characterized.

ALA-based photodynamic therapy has been investigated to enhance treatment for non-small cell lung cancer.[12] The results from clinical trials for basal cell carcinoma and recurrent superficial bladder carcinoma demonstrated that ALA-mediated photodynamic resulted in less discomfort and pain without skin photosensitivity and systematic toxicity.[13,14] Furthermore, ALA has been used clinically in tumor diagnosis and for photodynamic therapy of skin, brain, and bladder cancer.[15] Thus, we investigated the effects of ALA on HbF production and its potential to be developed as a therapeutic agent for the treatment of β-hemoglobinopathies.

Using the KU812 erythroid cell line, we showed that ALA induced γ-globin expression. Subsequently, we confirmed ALA-induced HbF activation at both transcriptional and translational levels in human primary erythroid progenitors generated from $CD34^+$ stem cells. Mechanistic studies demonstrated that the heme biosynthesis pathway was essential for ALA-induced γ-globin activation with simultaneous production of reactive oxygen species (ROS). Our results suggest that ALA induces HbF production through heme-regulated and ROS-mediated mechanisms.

Materials and methods

Antibodies and chemical agents

Antibodies for HbF (sc-21756), HbA (sc-21757), and NRF2 (sc-13032) were purchased from Santa Cruz Biotechnology (Dallas, TX) and Actin antibody (MAB1501) was purchased from EMD Millipore (Billerica, MA); TBP antibody (#8515S) was obtained from Cell Signaling (Danvers, MA). Chemical agents including ALA, succinylacetone (SA), acifluorfen (Acif), menadione (MND), tert-Butylhydroquinone (tBHQ), cysteine, and hemin were purchased from Sigma-Aldrich (St. Louis, MO).

Tissue culture

KU812 cells were maintained in Iscove's Modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), Penicillin (100 U/mL), and Streptomycin (0.1 mg/mL) with 4 mM L-glutamine at 37°C with 5% CO_2. KU812 cells were treated with ALA for 48 h unless otherwise indicated. The KU812 stable cell lines were established with a dual luciferase reporter plasmid containing a 3.1 kilobase cassette composed of the micro-locus control region (μLCR) and β-globin and ^{A}γ-globin gene proximal promoters[16] cloned upstream of the Renilla and Firefly luciferase genes, respectively. They were grown under the same conditions in the presence of G418 (800 μg/mL) as selection marker.

Human $CD34^+$ stem cells from healthy donors (Fred Hutchinson Cancer Research Center, Seattle, WA) were cultured in a two-phase liquid culture system as previously reported by our group.[17] During phase 1, cells were grown in IMDM with 15% fetal bovine serum, 15% human AB serum, 10 ng/mL interleukin-3, 50 ng/mL stem cell factor, and 2 IU/mL erythropoietin. Phase 2 was initiated on day 7 with IMDM, 15% fetal bovine serum, 15% human AB serum, and 2 IU/mL erythropoietin. ALA or hemin treatments at various concentrations with or without SA were conducted on day 8 and cells harvested on day 10 for mRNA levels and Western blot analysis.

Preparation of protein extracts

To prepare whole cell lysate samples, cells were lysed in a lysis buffer.[18] Soluble cell lysates were collected. To prepare nuclear and cytosolic protein extracts, a previously published procedure was used.[19] Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA).

Reverse transcription-quantitative polymerase chain reaction

Total RNA was extracted from cells using RNA Stat-60 (TEL-TEST "B" Inc., Friendswood, TX) per manufacturer's instruction. To determine levels of gene transcription, reverse transcription (RT) was performed with 1 μg RNA using the Improm-II reverse transcriptase system (Promega, Madison, WI) using $Oligo(dT)_{15}$ as primer. Complementary DNA generated in the RT reaction was mixed with iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA) and gene-specific primers (listed in Supplemental Table S1) as previously reported[20] and quantitative polymerase chain reaction (qPCR) was performed on the iCycler iQ system (Bio-Rad). Transcription levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-Actin (ACTB) were used as internal controls. The $2^{-\Delta\Delta CT}$ method[21] was applied to analyze target gene transcription levels normalized to levels of internal control genes and fold change of target gene transcripts in treated samples verses untreated control samples calculated.

ELISA to detect cellular hemoglobin levels

HbF and total hemoglobin (Hb) levels were quantified using the human Hemoglobin F and human Hemoglobin ELISA Quantitation kits, respectively (E80-136 and E80-135, Bethyl Laboratories). Briefly, whole cell lysates were added to plates coated with capture antibody (A80-136A for hemoglobin F and A80-134A for total hemoglobin) and incubated at room temperature for 1 h. After washing, respective horseradish peroxidase-conjugated detection antibody (A80-136P for hemoglobin F and A80-134P for total hemoglobin) was added and then unbound detection antibody was removed by wash. The chemical reaction was stopped by addition of 0.18 M sulfuric acid. The absorbance at 450 nm was determined using a Synergy MX microplate reader (BioTek). Hemoglobin F and total hemoglobin concentration were determined using respective standard curves produced from protein standards by the manufacture. The relative HbF levels were calculated using a procedure published previously.[18]

Western blot analysis

Western blot analysis was performed as previously published.[22] Whole cell lysates or nuclear proteins were resolved on sodium dodecyl sulfate-polyacrylamide gel followed by electro-transferring to a nitrocellulose membrane followed by incubation with antibodies specific for HbF (1:1000 dilution), HbA (1:1000 dilution), Actin (1:5000 dilution), TBP (1:2000 dilution), or NRF2 (1:1000 dilution). Membrane was washed with Tris-buffered saline with Tween-20 (TBS-T); horseradish peroxidase-conjugated goat anti-mouse (1:20,000 dilution, 31430, Thermo Fisher Scientific) or goat anti-rabbit antibody (1:20,000 dilution, 31460, Thermo Fisher Scientific) solution was used as the secondary antibody. ECL™ prime was used to detect the presence of protein. Images were captured on a Chemi-Doc MP Imaging System (Bio-Rad, CA).

Quantitative heme assay

The total cellular heme content was determined using the QuantiChrom™ Heme Assay Kit (DIHM-250, BioAssay Systems, Hayward, CA) per the manufacturer's instructions. Briefly, cellular lysate (25 µL) was mixed with 100 µL of detection reagent. Incubation was carried out at room temperature for 5 min followed by measuring the absorbance at 400 nm on a Synergy MX microplate reader by BioTek (Winooski, VT). The total heme concentration was calculated based on the formula: Heme concentration $= (OD_{sample} - OD_{blank})/(OD_{calibrator} - OD_{blank}) \times 125 \times$ dilution factor. This value was normalized by the total protein in each sample.

Detection of ROS

ROS levels were measured as previously published.[23,24] Cells were incubated with 2 µM 2′-7′-Dichlorodihydrofluorescein diacetate (DCFH-DA, Invitrogen, Carlsbad, CA) and green fluorescent intensity quantified using the LSR-II (BD Biosciences, CA) flow cytometer.

Dual luciferase assay

The KU812 dual luciferase reporter stable cells were plated at a density of 1.0×10^6 cells/mL in IMDM supplemented with 10% fetal bovine serum and G418 (800 µg/mL). Drug treatments were conducted with 10 µM cysteine (negative control), 50 µM hemin (positive control), and 0.2 mM, 0.5 mM, and 2 mM ALA for 48 h. Luciferase activity was measured using the Dual Luciferase Assay Reporter system (Promega, Madison, WI) as previously published.[16]

ChIP assay

Chromatin immunoprecipitation (ChIP) assay was performed as previous published.[17] Immunoprecipitation was performed with control IgG or NRF2 antibody (SC-13032x, Santa Cruz Biotechnology, CA) using the EZ-ChIP assay kit (Millipore, MA). Enrichment of chromatin DNA was detected by qPCR using primers specific for the antioxidant response element (ARE) in the proximal γ-globin and NQO1 promoters[25] (Supplemental Table S1). Chromatin enrichment was determined by qPCR analysis and relative levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Flow cytometry analysis of HbF-positive erythroid progenitor cells

To measure the HbF positive cells (%F-cells), erythroid progenitors were fixed with 1% formaldehyde and stained with fluorescein isothiocyanate (FITC) anti-HbF antibody (ab19365, Abcam); isotype control IgG antibody (MBS524511, MyBioSource, San Diego, CA) was used to detect non-specific staining. The %F-cells levels and mean fluorescence intensity (MFI) of F-cells were analyzed on LSR-II flow cytometer (BD Biosciences, San Jose, CA).

Statistical analysis

Each experiment conducted in triplicate for every experimental condition was repeated for at least three independent replicates. The fold change in gene or protein expression was calculated and represented as Mean ± standard error of the mean (SEM). Statistical analyses of the results were performed using unpaired Student's *t*-test, one-way analysis of variance (ANOVA) with Tukey's Multiple Comparison Test, or two-way ANOVA; $P < 0.05$ was considered statistically significant.

Results

ALA activates HbF synthesis in the human KU812 cell line

ALA has been shown to accelerate heme production,[10] enhance globin mRNA translation, and increase globin chain synthesis in cell culture systems.[6,11] Previously, we demonstrated that the fetal γ-globin and adult β-globin genes are actively transcribed in KU812 cells[26] and responsive to various HbF-inducing agents. These findings support using KU812 cells as a model to investigate the effects of ALA on HbF induction and mechanisms involving heme biosynthesis. KU812 were treated with ALA at different

doses for 48 h and total RNA and whole cell lysates prepared for determining γ-globin and β-globin gene transcription by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and HbF levels by Western blot. Treatment with 0.2 mM to 2 mM ALA produced >2-fold activation of γ-globin transcription, whereas β-globin mRNA was not significantly changed (Figure 1(a)). We next performed studies to determine the time course of γ-globin activation, which occurred by 6 h of ALA treatment ($P < 0.05$) and reached a maximal 2.2-fold ($P < 0.001$) induction at 48 h (Figure 1(b)). Interestingly, Western blot analysis showed that ALA increased HbF protein levels as early as 4 h (Figure 1(c)), which preceded γ-globin activation. As a second method to quantify HbF levels, enzyme-linked immunosorbent assay (ELISA) was conducted, which detected a dose-dependent 3.5-fold increase in HbF levels (Figure 1(d)). These results indicate that ALA treatment promotes γ-globin expression at both the transcriptional and translational levels in KU812 erythroid cells.

Heme biosynthesis involved in mechanisms of γ-globin activation by ALA

Knowing that exogenous ALA treatment rapidly activates γ-globin gene transcription, we asked what role heme biosynthesis plays in HbF induction. The synthesis of cellular heme is initiated by ALAS to produce ALA in the mitochondrion (Figure 2(a)). Two forms of ALAS exist including the ubiquitously expressed ALAS1 and erythroid-specific ALAS2 encoded by different genes; subsequent steps of heme biosynthesis are catalyzed by seven enzymes across mitochondrion and cytoplasm. To determine the relevance of heme synthesis in HbF induction by ALA, we inhibited this pathway using SA and Acif targeting ALA dehydratase (ALAD) and protoporphyrinogen oxidase (PPOX), respectively (Figure 2(a)).

We first studied the ability of ALA to increase heme levels. KU812 were treated with 0.5 mM and 2 mM ALA for 48 h, where a 1.5-fold ($P < 0.001$) increase in cellular heme was produced (Figure 2(b)). As shown in Figure 2 (c), SA pretreatment repressed the ability of ALA to induce γ-globin transcription and HbF expression. Likewise, Acif pretreatment blocked γ-globin activation by ALA as well (Figure 2(d)). These results support an essential role of the heme biosynthesis pathway in γ-globin activation by ALA and that integrity of this pathway is critical for mediating HbF production.

We next performed studies to substantiate the ability of exogenously added hemin to activate γ-globin transcription and to rescue SA-mediated suppression of γ-globin

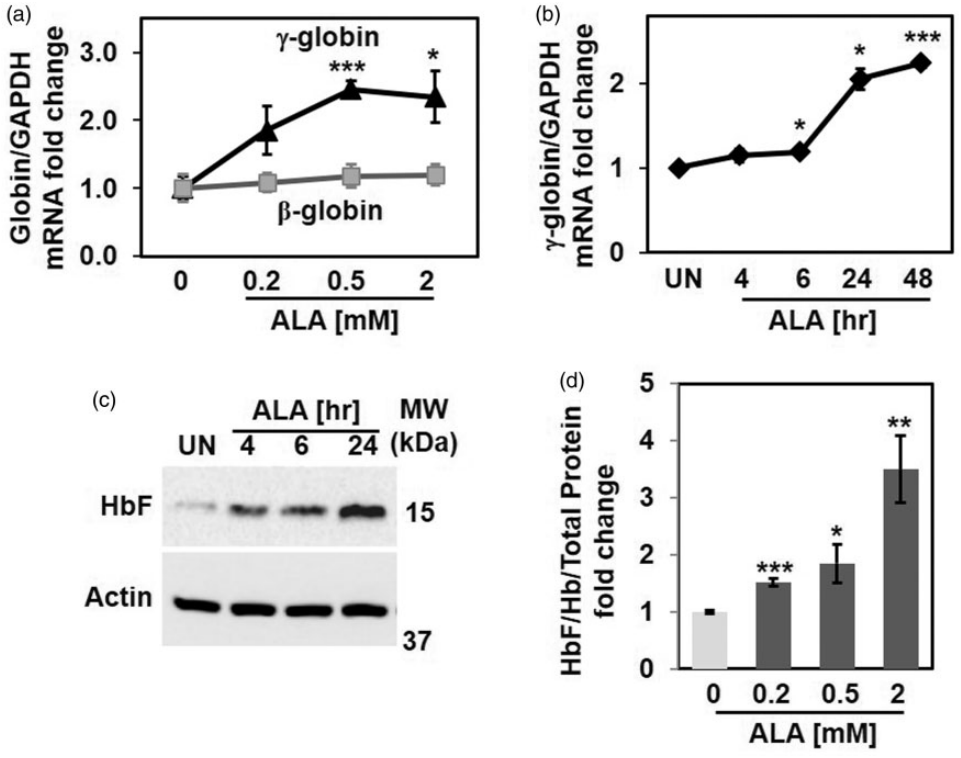

Figure 1. ALA treatment induces HbF production in erythroid cell line. KU812 cells were treated with ALA at the indicated concentrations or times and RNA and whole cell protein extracts were prepared for RT-qPCR, ELISA, and Western blot analysis (see Materials and methods section). The quantitative results in figures are shown as the Mean ± SEM from independent experiments with triplication of each experimental condition; a P value <0.05 was considered statistically significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$. (a) Dose response experiments were performed to determine the effect of ALA on globin gene transcription. RT-qPCR analysis quantified mRNA transcripts for γ-globin, β-globin, and GADPH. The fold changes of mRNA levels for γ-globin/GAPDH (dark triangle, $n = 5$) and β-globin/GAPDH (gray square, $n = 5$) are shown. (b) Time-course experiments determined effects of 1 mM ALA on γ-globin transcription in KU812 cells. Shown are fold changes for γ-globin/GAPDH mRNA ($n = 5$). (c) Western blot analysis was performed with anti-HbF antibody. Shown is a representative blot for KU812 cells treated with 1 mM ALA; Actin was used as internal control. (d) ELISA results demonstrated a dose-dependent increase of HbF protein in cells treated with ALA. ELISA assay was performed as described in Materials and methods section and fold change results are shown ($n = 5$). ALA: δ-aminolevulinic acid; HbF: fetal hemoglobin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; mRNA: messenger RNA; UN: untreated.

activation by ALA. As shown in Figure 3(a), ALA induced γ-globin transcription in KU812 cells by 3.3-fold which was inhibited by SA pretreatment. Addition of hemin partially reversed the inhibitory effects of SA on ALA treatment and achieved approximately 2-fold increase in γ-globin transcription. Similar studies with hemin alone show a 1.4-fold γ-globin activation; of note, treatment with SA alone repressed γ-globin transcription. Using KU812 allowed us to analyze the effects of the same treatments on β-globin gene expression (Figure 3(b)). Hemin induced β-globin transcription 13.6-fold; however, SA pretreatment did not inhibit activation. By contrast, ALA had no significant effect on β-globin gene transcription suggesting hemin mediated a broader effect on globin gene regulation.

Since extracellular heme can trigger oxidative stress, which can be toxic to cells at high levels, we compared the effects of ALA and hemin on cell viability and proliferation using trypan blue exclusion. KU812 cells were treated with both agents and cell counts and viability determined at 24, 48, and 72 h. ALA (1–5 mM) produced minimal effects on cell numbers with >90% viable cells at all concentrations (Figure 3(c)). By contrast, hemin greatly inhibited cell proliferation; however, cell viability remained >90% except at the 40 μM concentration (Figure 3(d)). These results indicate that ALA and hemin exhibit different effects on cell growth and viability and have similar but different cellular mechanisms.

ALA mediates γ-globin activation via generation of ROS

It has been reported that free heme can trigger oxidative stress in cells and tissues[27,28] through protein aggregation and lipid oxidization. Therefore, we questioned whether ALA-stimulated heme synthesis promotes ROS production to stimulate the antioxidant response and NRF2 expression.[29–31] Using DCFDA staining and flow cytometry analysis, we measured ROS production after ALA treatment; MND, a potent inducer of ROS was used as a positive control.[32,33] For quantitative analysis, untreated cells were set

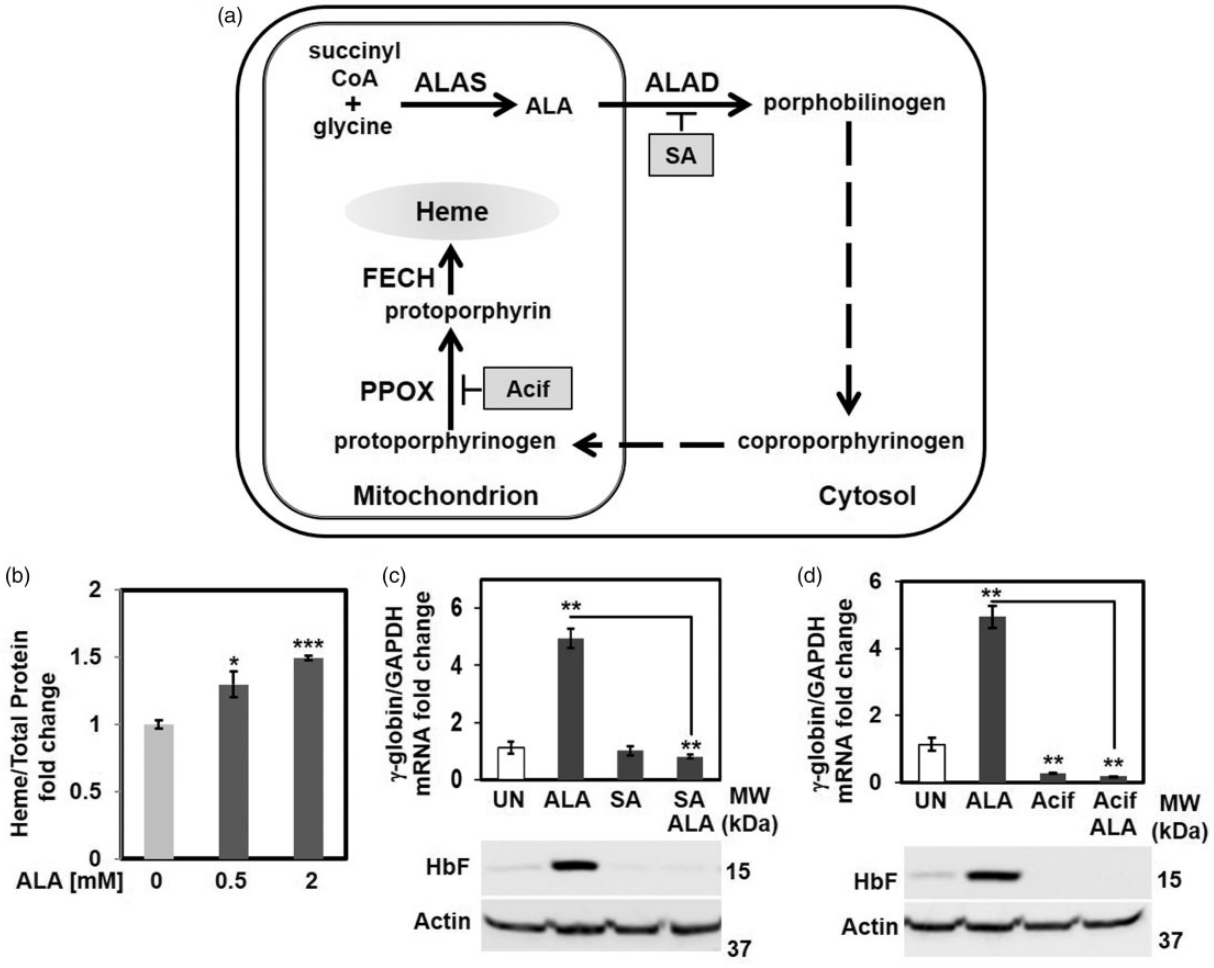

Figure 2. Heme biosynthesis is required for γ-globin activation by ALA. (a) A schematic of the heme biosynthesis pathway involving eight enzymes. ALAS produces ALA from succinyl CoA and glycine. Pharmacological pathway inhibitors including SA for ALAD and Acif for PPOX are indicated. (b) Heme levels were determined in KU812 cells treated with ALA at indicated concentrations for 48 h (see Materials and methods section). The fold changes of heme levels are shown ($n = 3$). (c) KU812 cells were pretreated with 1 mM SA for 30 min followed by the addition of 1 mM ALA for 48 h; total RNA and whole cell lysates were prepared for RT-qPCR and Western blot, respectively. Fold changes for GAPDH normalized mRNA are shown (top panel, $n = 5$); Western blot images for the protein levels of HbF and Actin for the same treatment (bottom panel). (d) RT-qPCR data for mRNA fold change are shown (top panel, $n = 5$) and Western blot images for HbF and Actin for the indicated treatment are shown (bottom panel). ALAD: δ-aminolevulinate dehydratase; ALAS: δ-aminolevulinate synthase; SA: succinylacetone; FECH: ferrochelatase; PPOX: protoporphyrinogen oxidase; Acif: acifluorfen; HbF: fetal hemoglobin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

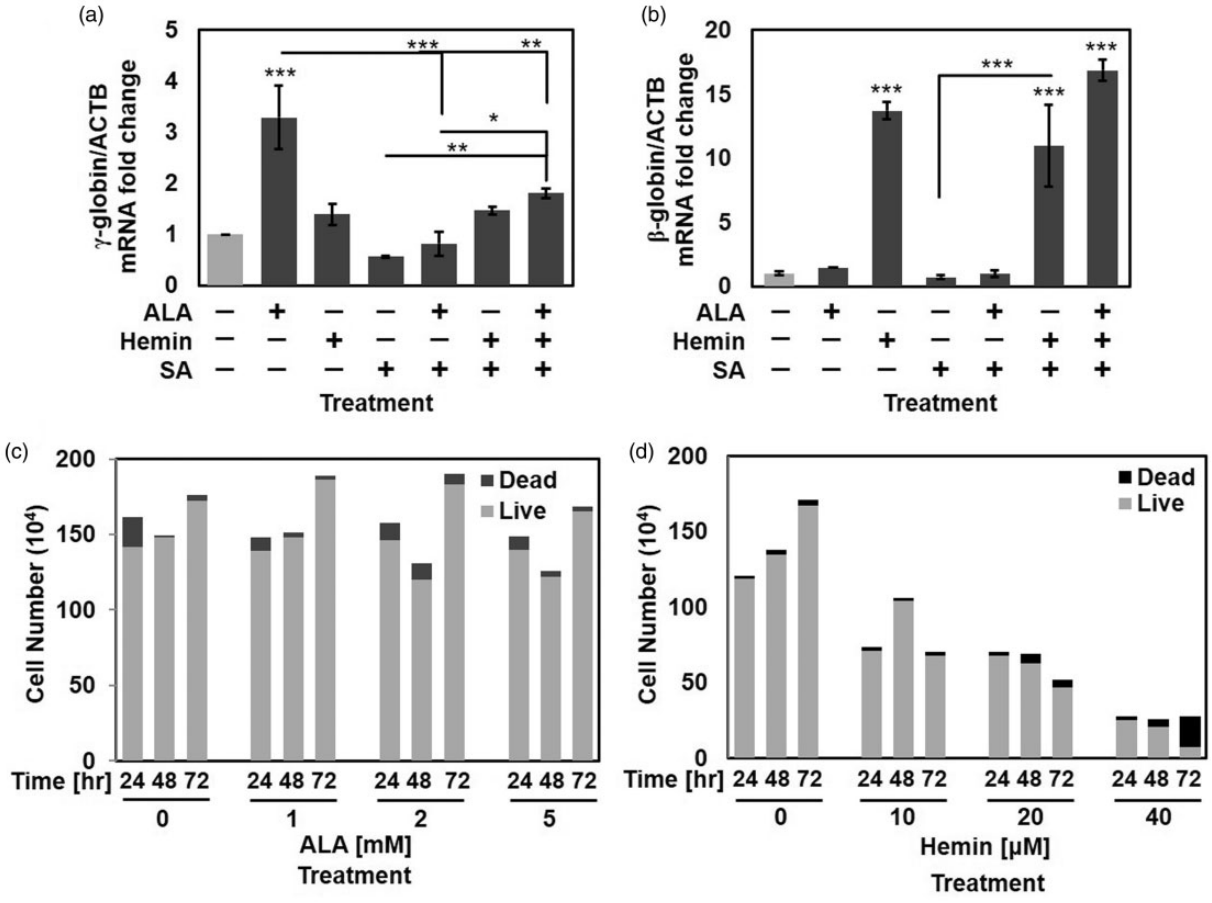

Figure 3. Hemin partially reverses the ability of SA to silence γ-globin activation by ALA. KU812 cells were treated with indicated reagents for 48 h in the absence or presence of SA pretreatment. For hemin rescue experiments, cells were pretreated with SA for 30 min followed by addition of ALA; after 24 h, hemin was added and cells harvested at 48 h. RT-qPCR analysis was conducted to determine the effect of indicated reagents on transcription of γ- and β-globin as well as ACTB. Quantitative results are shown as Mean ± SEM from independent experiments. Statistically analysis of data was performed using one-way ANOVA with Tukey's Multiple Comparison Test with GraphPad Prism 5.0 software. (a) Fold changes for γ-globin/ACTB mRNA are shown ($n = 5$). (b) Fold changes for β-globin/Actin mRNA are shown ($n = 5$). (c) KU812 cells were treated with increasing ALA concentrations for 72 h. Cell growth and viability was monitored by trypan blue exclusion. Average cell counts during the treatment are shown ($n = 3$). (d) Using a similar design, KU812 cells were treated with hemin at the concentrations shown and cell growth and viability were monitored by trypan blue exclusion. The data were analyzed as described above ($n = 3$). ALA: δ-aminolevulinic acid; ACTB: β-actin; SA: succinylacetone; mRNA: messenger RNA.

at background threshold fluorescence. Figure 4(a) is a representative composite image of histograms for KU812 cells with indicated treatments. As expected, MND produced a right shift in the histogram and 2.1-fold burst of ROS production (Figure 4(b)); likewise, ALA increased ROS 1.7-fold ($P < 0.001$). In contrast, SA alone or SA combined with ALA treatment significantly decreased ROS levels by about 55% ($P < 0.001$). These results suggest that ALA increases ROS production through heme biosynthesis, which contributes to HbF induction (Figure 2).

To confirm the role of ROS production in HbF induction by ALA, we treated cells with dimethylthiourea (DMTU), a hydroxyl radical scavenger. As shown in Figure 4(c), DMTU significantly reduced ROS levels by >50% ($P < 0.05$) in the absence or presence of ALA treatment. Moreover, DMTU inhibited ALA-induced γ-globin gene transcription (Figure 4(d)) and abolished HbF induction by ALA as shown by representative Western blots (Figure 4(e)) and ELISA results (Figure 4(f)). These results confirm that cellular oxidative stress, triggered by ALA-stimulated hydroxyl radicals, is required for γ-globin gene activation.

ALA preferentially activates the γ-globin promoter through NRF2 binding *in vivo*

Results in Figure 1(b) suggest that ALA preferentially activates γ-globin transcription. Therefore, to determine whether ALA affects γ-globin transcription through increasing promoter activity and transcription factor binding, we utilized a KU812 stable cell line expressing a dual-luciferase cassette comprised of the Firefly reporter upstream of γ-globin (γ-Firefly) and the Renilla reporter upstream β-globin (β-Renilla) (Figure 5(a)); treatments with cysteine and hemin were used as negative and positive controls, respectively. Figure 5(a) shows that ALA induced γ-Firefly luciferase activity in a dose-dependent manner up to 5-fold ($P < 0.001$), similar to the 5.5-fold induction by hemin. Interestingly, although ALA did not increase β-globin promoter activity, hemin increased β-Renilla by 1.8-fold. Further analysis of the combined

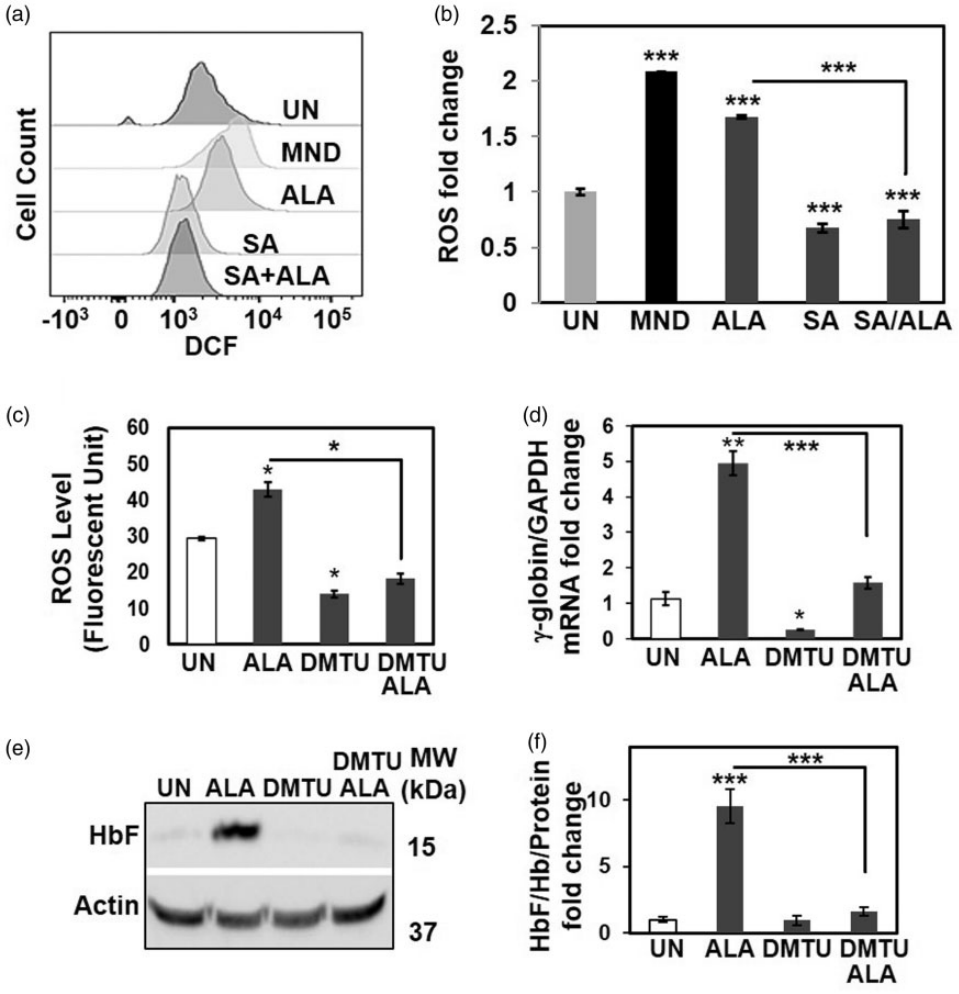

Figure 4. ROS induced by ALA contribute to γ-globin activation. KU812 cells were treated as indicated and flow cytometry analysis performed to measure ROS levels using 2′,7′-Dichlorodihydrofluorescein diacetate DCFH-DA as a probe (see Materials and methods section). Quantitative results are shown as Mean ± SEM from independent experiments. (a) Shown are the overlaid histograms with DCF fluorescent signal on the *x*-axis and cell count on the *y*-axis. (b) Shown in the graph are the fold changes of ROS levels ($n = 3$) with indicated reagents. (c) KU812 cells were pretreated with 40 mM DMTU, a hydroxyl radical scavenger, for 30 min followed by ALA for 48 h. Fold changes on ROS levels are shown ($n = 5$). (d) RNA samples prepared from KU812 cells treated with reagents indicated in panel (c) were analyzed by RT-qPCR. Fold changes for γ-globin/GAPDH mRNA are shown ($n = 5$). (e) Western blot analysis determined HbF expression with actin as internal control; representative blots are shown. (f) ELISA analysis determined HbF complex levels. Results were normalized with total hemoglobin and protein input. Fold changes are shown ($n = 5$). DCF: dichlorofluorescein; DMTU: dimethylthiourea; ROS: reactive oxygen species; SA: succinylacetone; ALA: δ-aminolevulinic acid; HbF: fetal hemoglobin; MND: menadione; UN: untreated.

effects on both globin promoters shown as the $\gamma/(\gamma+\beta)$ ratio, confirmed a 1.8-fold increase by 2 mM ALA, which was comparable to hemin treatment. These results suggest that both ALA and hemin increase γ-globin promoter activity.

We then asked what transcription factor(s) contributed γ-globin gene activation by ALA. It has been shown that transcription factor NRF2 is a major regulator of the cellular antioxidant response.[34] Furthermore, NRF2 is known to transactivate the γ-globin promoter through binding to the ARE[25] present in the proximal γ-globin promoter. Oxidative stress produced by cellular ROS triggers NRF2 protein stabilization by promoting proteasome-mediated degradation of KEAP1 (NRF2 inhibitor) and promoting nuclear localization.[35] Therefore, we quantified NRF2 mRNA levels along with its target genes to determine the effects of ALA-induced ROS on the cellular antioxidant response. As shown in Figure 5(b), ALA produced a dose-dependent increase in NRF2 transcription up to 1.7-fold with subsequent activation of downstream targets NAD(P)H dehydrogenase (quinone) 1 NQO1 and heme oxygenase 1 (HMOX1) (Supplemental Figure S1). Additionally, we observed an increase in nuclear NRF2 levels and HbF induction by Western blot supporting ALA-activated NRF2 nuclear localization and its role as positive regulator for γ-globin transcription (Figure 5(c)).

Since NRF2 is known to transactivate the γ-globin promoter through binding the proximal ARE in cells treated with tBHQ, an antioxidant,[36] and we observed NRF2 activation by ALA, we asked the question whether this mechanism is involved in γ-globin activation. Thus, we performed ChIP assay to quantify NRF2 binding *in vivo*. We detected a 9-fold increase in NRF2 binding in the γ-globin ARE after 2 mM ALA treatment, which was reversed by SA pretreatment (Figure 5(d)). Likewise, we observed robust 75-fold increase in NRF2 binding in the

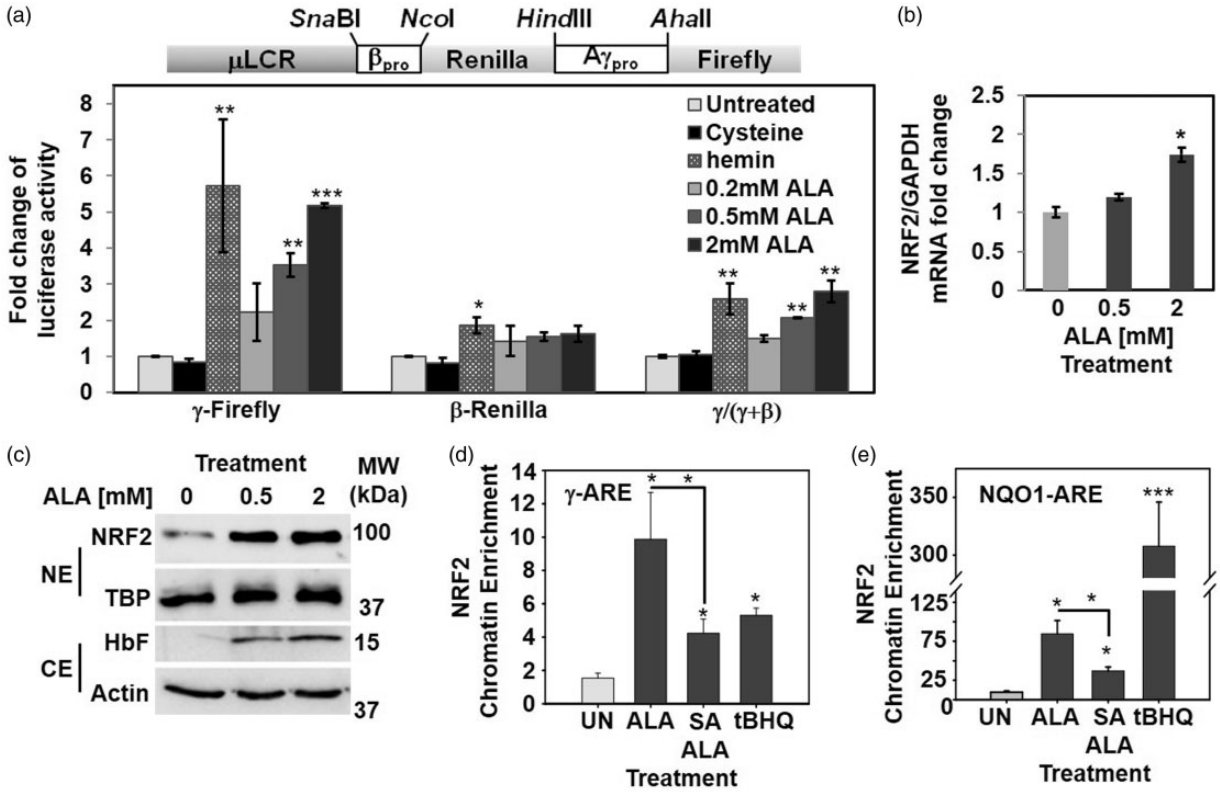

Figure 5. ALA preferentially activates γ-globin promoter and induces NRF2 binding *in vivo*. (a) Shown at the top is a schematic of the dual luciferase plasmid construct used in these experiments. The Renilla luciferase gene is controlled by a 300 bp β-globin proximal promoter (β_{pro}), whereas the Firefly luciferase gene is driven by a 1.5 kb Aγ-globin promoter ($A\gamma_{pro}$). A 4 kb μLCR was cloned upstream of the β_{pro}. Cells were treated with 10 μM cysteine (negative control), 50 μM hemin (positive control), and 0.2 mM, 0.5 mM, and 2 mM ALA for 48 h. Luciferase activities were measured using a dual-luciferase Reporter Assay System (see Materials and methods section). Shown are the fold changes in luciferase activity ($n = 2$). Firefly luciferase activities representing the γ-globin promoter (γ-Firefly) are shown on the left; Renilla luciferase activities representing the β-globin promoter in the middle (β-Renilla); the $\gamma/(\gamma+\beta)$ ratio is shown on the right. (b) ALA activated NRF2 transcription. RNA samples were prepared from KU812 cells treated with ALA at indicated concentration for 48 h. RT-qPCR analysis was performed. Fold changes for NRF2/GAPDH mRNA are shown ($n = 3$). (c) CE and NE were prepared from KU812 cells and Western blot performed for NRF2, TBP, HbF, and Actin. Representative blots are shown indicating ALA increased nuclear NRF2 and cytosol HbF levels. (d) ChIP assay was performed using NRF2 antibody and IgG as control for immunoprecipitations; chromatin enrichment at the γ-globin ARE was determined by qPCR and normalized to input chromatin and IgG control. Results from two independent experiments are shown and two-way ANOVA performed ($n = 2$). (e) ChIP assay was conducted for the ARE in the NQO1 promoter and analyzed as described in panel (d) ($n = 2$). μLCR: micro locus control region; NE: nuclear extracts; CE: cytosolic protein samples; ALA: δ-aminolevulinic acid; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; mRNA: messenger RNA; SA: succinylacetone; ARE: antioxidant response element; BHQ: butylhydroquinone.

NQO1 promoter after ALA treatment (Figure 5(e)); the positive control tBHQ produced a 300-fold increased NRF2 binding ($P < 0.0001$). The inhibition of NRF2 binding in both promoters by SA supports a role of ALA-mediated heme biosynthesis and implicates the involvement of NRF2 binding in the ARE in the molecular mechanism of γ-globin gene activation.

ALA activates γ-globin expression in primary human erythroid progenitors

Although KU812 provides us an excellent cellular model to study γ-globin expression with limited β-globin transcription, these cells lack the regulatory features of normal primary erythroid cells. Therefore, we used human erythroid progenitors derived from $CD34^+$ stem cells in a two-phase liquid culture system established in our lab[17] to confirm the effects of ALA treatment on globin gene expression. Erythroid progenitor cells were cultured described in Materials and methods section and treatments with various agents were conducted at day 8 for 48 h. Subsequently, total RNA and whole cell lysates were prepared for RT-qPCR and Western blot analyses. RT-qPCR results in Figure 6(a) show that ALA preferentially stimulated γ-globin transcription by 2-fold, which was significantly inhibited by SA pretreatment. By contrast, ALA had no effect on β-globin and α-globin gene transcription, or genes encoding CD71 (transferrin receptor) and CD235a (glycophorin A), two important cell surface markers of erythroid differentiation. On the other hand, hemin treatment induced γ-globin transcription by 4-fold and activated β-globin transcription 2-fold; however, neither ALA nor hemin significantly affected α-globin transcription. These results support preferential γ-globin gene activation by ALA in human primary erythroid progenitors.

To demonstrate that activation of γ-globin transcription by ALA translates into increased HbF expression, we conducted Western blot analysis. As shown in Figure 6(b) and (c), 2 mM ALA induced HbF 2.2-fold that reversed after SA pretreatment. Similar to mRNA data, ALA did not change HbA levels significantly. On the other hand,

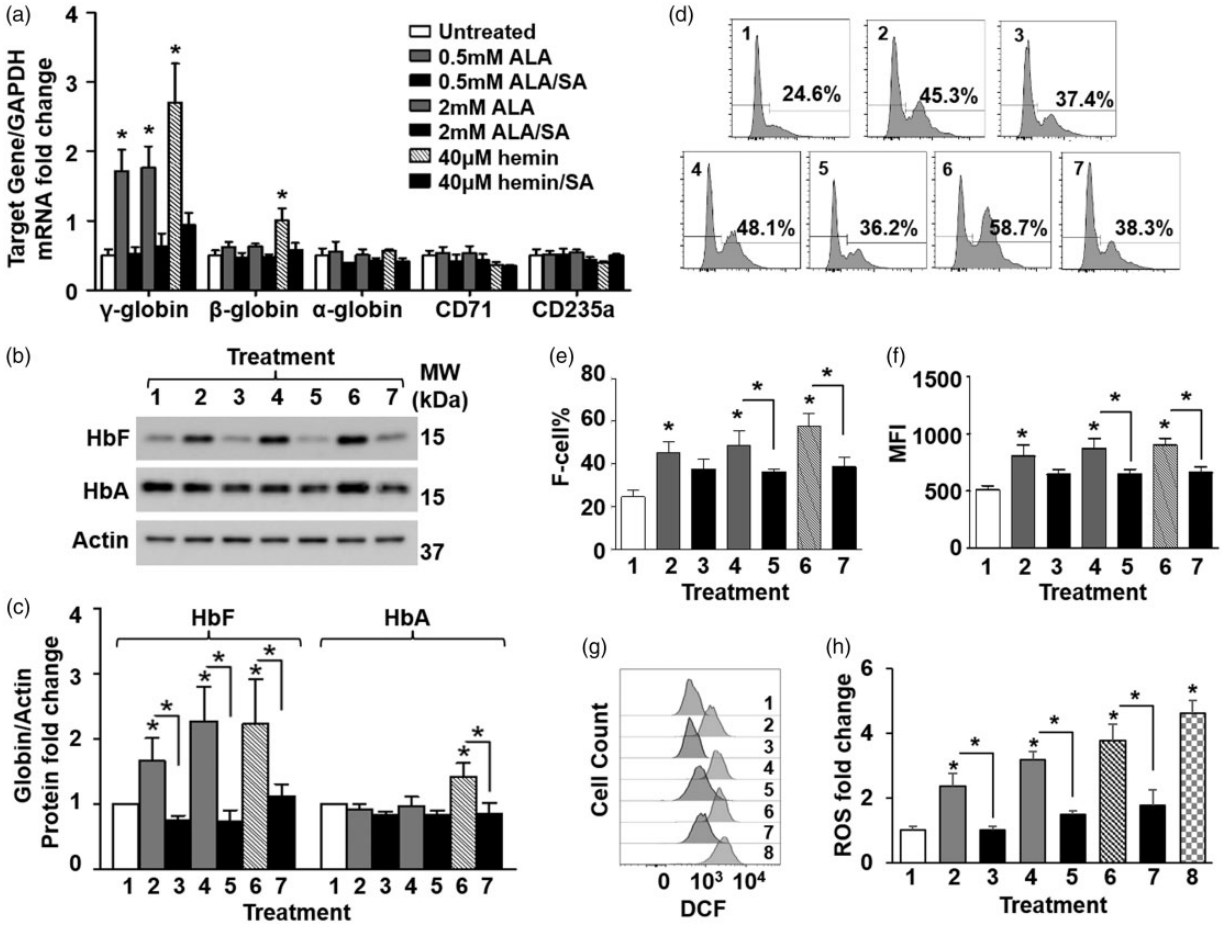

Figure 6. ALA induces γ-globin gene expression in normal human erythroid progenitors. Erythroid progenitors generated from $CD34^+$ stem cells (see Materials and methods section) were treated at day 8 for 48 h with different concentrations of ALA with or without SA and hemin as a positive control. Treatment conditions: Untreated (1), 0.5 mM ALA (2), 0.5 mM ALA with SA (3), 2 mM ALA (4), 2 mM ALA with SA (5), 40 mM hemin (6), 40 mM hemin with SA (7), and MND (8). Quantitative results are shown as Mean ± SEM from independent experiments. (a) RT-qPCR was conducted to determine the mRNA levels for the genes shown. The expression of gene in untreated erythroid progenitors was set to one after normalized to the GAPDH mRNA levels. The quantitative data are shown ($n = 3$). *$P < 0.05$. (b) Western blot analysis was conducted for the protein shown with whole cell lysates isolated from erythroid progenitors with the indicated agents. Shown are representative images. (c) The relative protein expression was quantified by densitometry with Actin as the loading control. Results are shown ($n = 3$). *$P < 0.05$. (d) Flow cytometry analysis was conducted to determine the %HbF positive cells (%F-cells) stained with FITC-labeled anti-HbF antibody. Representative histograms for the F-cell staining are shown ($n = 3$). (e) Quantitative data with %F-cell are shown. (f) Mean fluorescence intensity results for the flow cytometry analysis are shown ($n = 3$). (g) ROS levels after ALA or hemin treatment in the same cells were determined. Representative histograms are shown. Numeric labels on the right indicate treatment conditions define above. MND treatment was used as a positive control agent for ROS induction. (h) Quantitative ROS data are shown ($n = 3$). *$P < 0.05$. SA: succinylacetone; ALA: δ-aminolevulinic acid; DCF: dichlorofluorescein; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HbF: fetal hemoglobin; mRNA: messenger RNA.

hemin treatment increased HbF (2.0-fold) and HbA (1.3-fold) levels. To determine the cellular distribution of HbF in erythroid progenitors, we performed flow cytometry analysis using FITC-conjugated anti-HbF to quantify the percentage of HbF positive cells (F-cells). Figure 6(d) shows representative histograms of flow cytometry for different treatment conditions demonstrating an increase in F-cells by ALA and hemin. Quantitative analysis of flow cytometry data is shown in Figure 6(e). Both ALA and hemin treatment produced approximately 2-fold increases of F-cells, from 23% to 48% by 2 mM ALA and 23% to 52% by hemin. Lastly, we measured by flow cytometry the MFI (See Material and Methods), which is a quantitative measure of HbF protein per cell in FITC-stained erythroid progenitors. As shown in Figure 6(f), we observed 1.7-fold increase of HbF by both 2 mM ALA and hemin. These results confirm that ALA mediated γ-globin activation at the transcriptional and translational levels in human primary erythroid progenitor.

ALA increase ROS levels in primary human erythroid progenitors

In KU812 cells, we demonstrated that ALA induced ROS production, which activated NRF2 expression involved in established mechanisms of γ-globin gene regulation.[17,24,25] We therefore asked whether ALA and hemin treatments produced similar effect on ROS production in primary erythroid progenitors. Figure 6(g) depicts representative histograms for ROS levels detected by flow cytometry. A shift of peaks to the right after ALA and hemin treatment was observed supporting increased ROS production, whereas SA pretreatment abolished this effect. Quantitative data shown in Figure 6(h) confirm increased ROS production

at 3.2-fold and 3.8-fold by 2 mM ALA and hemin treatment, respectively.

Discussion

By the 1990s, it was demonstrated that elevated HbF levels improves long term survival in adults with SCD[37] and with completion of the Multicenter Study of Hydroxyurea Trial, HU became the first Food and Drug Administration-approved HbF inducing agent with proven clinical efficacy in SCD.[1] However, a significant number of individuals do not respond to therapy. Therefore, research efforts have continued using cell culture systems[35,38–40] and transgenic animal models[35,41] to develop additional effect agents to induce HbF. Parallel drug development efforts progress continues toward two curative therapies for SCD including hematopoietic stem cell transplantation and gene therapy.

In our study, we investigated a small molecule ALA, the first intermediate chemical produced during heme synthesis[42] as a potential HbF inducer. It is known that heme and globin synthesis are tightly coordinated events in erythroid cells to ensure balanced production of hemoglobin.[43] We demonstrated that ALA activated γ-globin transcription with negligible effects on β-globin expression in $CD34^+$-derived primary erythroid progenitors. Furthermore, enhanced heme biosynthesis and HbF induction occurred simultaneously in a dose-dependent manner. These results are consistent with reports showing that ALA induces hemoglobin production in various erythroid cell cultures, with minimal toxicity.[11,44,45] Kawasaki *et al*. reported that ALA augmented production of hemoglobin in K562 cells that was reduced by the addition of SA to block the heme biosynthesis pathway.[44] Using SA in our studies, we observed inhibition of HbF induction by ALA supporting a role of heme biosynthesis in the mechanism of γ-globin activation.

Interestingly, our time course studies showed enhanced HbF protein levels prior to the activation of γ-globin transcription suggesting enhanced mRNA translation by ALA. Similar mechanisms, reported in SCD patients treated with intermittent arginine butyrate,[46] demonstrated increased γ-globin chain synthesis within 24 h of treatment in the absence of increase γ-globin transcription through enhanced translation efficiency. Additional studies are required to verify this mechanism of action for ALA.

It is well known that heme binds and inactivates the stress response protein kinase, heme-regulated inhibitor (HRI),[47] which regulates phosphorylation of the translation initiation factor 2α (eIF2α) at serine 51 and permits global translation initiation. We recently reported that ALA treatment reduced phosphorylated eIF2α levels[48] supporting possible involvement of HRI/eIF2α signaling in HbF induction by ALA. More recently, Grevet *et al.* identifies HRI as a repressor for HbF in erythroid cells in a single-guide RNA library screen targeting 482 protein kinase domains.[49] They reported enrichment of HRI targeting single-guide RNAs in association with high HbF in erythroid cells, with concomitant decreased eIF2α phosphorylation. Subsequent HRI knockdown in human primary erythroid progenitors demonstrated γ-globin activation and HbF induction; furthermore, HRI knockdown produced anti-sickling effects in sickle erythroid progenitors. These findings provide additional support for modulating HRI as a strategy to induce HbF for the treatment of SCD. Since heme synthesis is stimulated by ALA and it is a natural inhibitor for HRI activity, whether this mechanism is involved in HbF induction requires additional studies.

We observed that hemin partially restores the ability of ALA to induce γ-globin after treatment with SA (to block heme synthesis), suggesting other mechanisms are involved in HbF induction by this agent. For example, heme binds to BACH1, a transcription repressor, to decrease BACH1 binding to ARE-like elements in the HMOX1 promoter and enhancer. This interaction removes BACH1 from regulatory elements and allows NRF2 binding to the ARE to activate HMOX1 transcription.[50] BACH1 and NRF2 belong to the basic leucine zipper family of transcription regulators and they form heterodimer with small MAF (sMAF) proteins to regulate target gene transcription.[51,52] Heme binding to BACH1 directly affects target gene transcription without NRF2 participation, such as induction of β-globin transcription by hemin.[8] On the other hand, thioredoxin reductase 1 transcription can be activated by NRF2 without involving BACH1[53] indicating diverse regulatory mechanisms provided by the interplay of the Heme-BACH1/NRF2/sMAF system. Our results support the ability of ALA and hemin to act through heme synthesis and NRF2 binding in the proximal promoter to activate γ-globin transcription.

To investigate this mechanism of γ-globin activation by ALA we confirmed NRF2 nuclear translocation that correlated with an increase in γ-globin transcription and HbF expression. We previously demonstrated that dimethyl fumarate induces HbF production by NRF2 activation, to facilitate long-range chromatin interactions.[17] Furthermore, in our novel SCD/NRF2 knockout mouse model, γ-globin expression was silenced during developmental erythropoiesis.[24] We show herein that ALA preferentially activates γ-globin promoter activity and enhanced *in vivo* NRF2 binding in the ARE. These data support a role of the ALA-ROS-NRF2 axis in activating γ-globin transcription, which are consistent with the SCD/NRF2 knockout mouse data.[24]

ALA and its derivatives are clinical therapeutic agents, for cancer diagnosis and treatment.[15,29] Light exposure after ALA administration produces activated fluorescent protoporphyrin IX provoking photodamage to tumor cells through ROS production. We observed an increase in ROS levels mediated by ALA to activate the cellular antioxidant response and HbF induction. The level of ROS generated by ALA is sufficient to activate NRF2, HMOX1, and NQO1 gene transcription suggesting activation of the cellular cytoprotection mechanism. Chronic hemolysis in many hematologic diseases including SCD results in release of extracellular heme and subsequent adverse effects such as inflammation and altered cell metabolism.[27] We observed increase in intracellular heme; however, whether plasma levels will change is beyond the scope of our mechanistic study, but it will be tested in future studies using SCD mice.

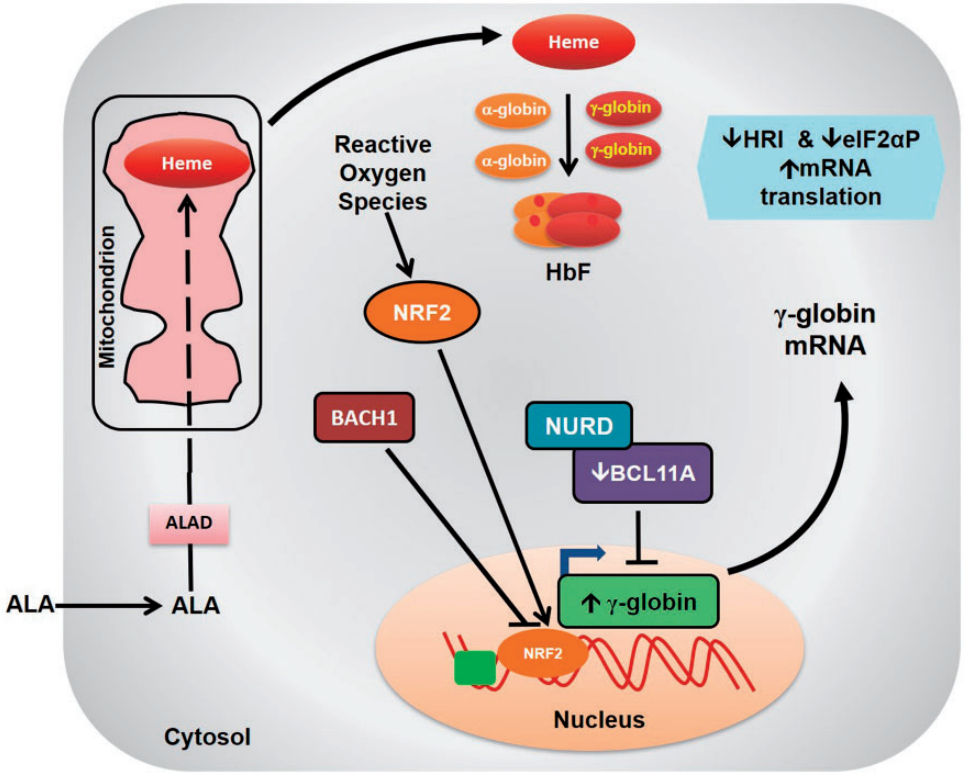

Figure 7. Model of ALA-mediated HbF induction. Collective data presented in this study and published reports, support our proposed model of γ-globin activation by ALA. Exogenous ALA stimulates heme biosynthesis, which binds globin chains to form functional hemoglobin molecules. In addition, heme binding to HRI suppresses expression to decrease eIF2α phosphorylation and enhance translational of globin mRNA transcripts.[47,55] Another potential downstream effect of heme-mediated HRI inactivation is the inhibition of BCL11A, a major repressor of γ-globin gene transcription.[49,56,57] ALA also increased ROS levels through stimulated heme biosynthesis, which triggers activation of NRF2 to promote nuclear translocation, binding in the ARE and enhanced γ-globin gene transcription. Therefore, the different effects of ALA and hemin on γ-globin regulation suggest that undetermined factors can be activated by heme to mediate γ-globin expression. HRI: heme-regulated inhibitor; mRNA: mRNA: messenger RNA; ALA: δ-aminolevulinic acid. (A color version of this figure is available in the online journal.)

Based on our results and the published reports, we propose a model of the effects of ALA on γ-globin expression shown in Figure 7. Exogenously administered ALA bypasses the rate-limiting enzyme ALAS and stimulates heme biosynthesis. Heme in turn represses HRI activity and reduces phosphorylated eIF2α, which promotes γ-globin mRNA translation and HbF synthesis. Through parallel mechanisms, ALA triggers ROS formation, and NRF2 activation to induce γ-globin transcription through binding the proximal ARE. Our data also suggest the involvement of other unidentified γ-globin regulators. Recently, we published a report demonstrating activation of HbF production by a prodrug conjugate AN-233 comprised of butyric acid and ALA in K562 cells and sickle erythroid progenitor cells.[54] Subsequent studies in preclinical β-YAC transgenic mice demonstrated HbF induction *in vivo*. This new report and results presented in current study support future investigations to test effects of ALA on HbF production in SCD mice and sickle erythroid progenitor cells.

Authors' contributions: LL designed and performed research, analyzed data, and wrote the paper. XZ performed the ChIP assay, analyzed the NRF2 enrichment data, conducted experiments, analyzed results for globin expression in human primary erythroid progenitors, and edited the paper. CMW contributed in dual luciferase assay and ROS studies. AY performed heme biosynthesis pathway experiments and edited the paper. BSP designed research, analyzed data, and edited the paper.

DECLARATION OF CONFLICTING INTERESTS

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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