Investigation of the role of heme oxygenase-1 in β-thalassemia pathophysiology

Arielle Mendel[1], Daniel Santos[2] and Prem Ponka[1,2]

1.Department of Physiology, McGill University, 3655 Promenade Sir William Osler, Montréal, Québec, H3G 1Y6
2.Lady Davis Institute for Medical Research, 3755 Côte Ste-Catherine Road, Montreal, Quebec H3T 1E2
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Abstract
β -thalassemia is an inherited disorder characterized by impaired hemoglobin synthesis in developing erythrocytes due to unbalanced globin chain production, resulting in severe oxidative damage to cells. Heme oxygenase-1 (HO-1) has been shown to have antioxidant benefits and is upregulated in response to oxidative stress; thus increased HO-1 levels could serve as an indicator of β-thalassemia pathophysiology. To test this hypothesis, we created a cell culture model of β-thalassemia by decreasing β globin expression in murine erythroleukemic (MEL) cells using short interfering RNA (siRNA). An increase in HO-1 protein expression, along with a decrease in heme levels was observed in cells where β globin reduction was sustained for 96 hours. Further investigation is needed to determine the role that HO-1 plays in normal erythroid development and confirm the observed relationship between β globin reduction and increased HO-1 expression, however, our results provide evidence that this method serves as an effective and novel cell model of β thalassemia.

Keywords
Erythroid cell, hemoglobin, heme oxygenase, β-thalassemia.

Glossary
Erythroid cell: cell undergoing differentiation to become an erythrocyte (red blood cell)
Hemoglobin: the hemoprotein responsible for binding and carrying oxygen in erythrocytes
Heme oxygenase: rate-limiting enzyme in the catalytic breakdown of heme, a component of hemoglobin
β thalassemia: erythrocyte pathology involving reduced β globin chain synthesis and impaired production of functional hemoglobin.

Introduction

Adult hemoglobin is primarily composed of a single heme moiety in a complex with two α globin and two β globin protein chains (Higgs, 1993). The expression levels of each component are tightly controlled in the developing erythrocyte to prevent accumulation of excess heme or globin (Ponka, 1999; Higgs, 1993). Heme is produced in the mitochondria of aerobic cells as a complex of iron (II) sitting at the centre of a macrocyclic compound, protoporphyrin IX. Specifically, heme production in erythroid cells is limited by the uptake of exogenous iron via transferrin receptors. Iron regulates heme biosynthesis by controlling the translation of erythroid-specific aminolevulinic acid synthase (ALA-S2), the first enzyme to act in the production of protoporphyrin IX.

During the production of hemoglobin, heme regulates globin at the transcriptional and translational levels and has been found to promote β-globin transcription by blocking the activity of the transcriptional repressor Bach1 during hemoglobin synthesis (Ponka, 1999; Tahara, et al. 2004). Additionally, heme upregulates NF-E2, an erythroid-specific transcription factor involved in globin gene activation (Ponka, 1999), and is required to inactivate the heme regulated inhibitor (HRI), which inhibits proteins involved in the initiation of globin translation by phosphorylation (Ponka, 1999; Chen, 2007). These mechanisms ensure that globin is produced proportionally to the intracellular concentration of heme.

Heme transcription and translation must be tightly maintained in all cells, since increased synthesis of heme above homeostatic levels can be highly toxic (Graca-Souza, *et al.* 2005). Heme oxygenase (HO) is a ubiquitous microsomal enzyme that is responsible for catalyzing the rate-limiting step in heme catabolism: the release of iron, carbon monoxide, and biliverdin (Ponka, 1999). Iron is then recycled within the body for subsequent hemoglobin synthesis in erythroid cells. While 85% of organismal heme synthesis occurs in immature erythroid cells, most heme catabolism takes place in splenic macrophages by the HO-1 isozyme (32 000 kDa) during the recycling of senescent erythrocytes (Ponka, 1999; Abraham, *et al.* 2008). The identification of multiple transcriptional inducers and response elements suggests a complexity that could allow multiple HO-1 activation pathways, each unique to different cell types, although it is unclear what role HO-1 plays in the developing erythrocyte. The heme molecule has been demonstrated to increase HO-1 expression through interaction with *Bach1* (Alam, *et al*. 2005), which would suggest that increased heme levels would result in increased heme degradation. However, heme must accumulate in high concentrations during erythroid development in order to produce hemoglobin, suggesting that the cell must have a regulatory mechanism that prevents heme degradation by HO-1. Furthermore, it is possible that HO-1 could be active in the erythroid cell under pathophysiological conditions relating to hemoglobin synthesis, such as β thalassemia.

β thalassemia is characterized by decreased synthesis of β globin chains during erythroid differentiation, usually due to a mutation in the β globin gene that may impair transcription, mRNA processing, or translation. As a result, balanced hemoglobin synthesis and overall erythropoeisis are hampered, producing microcytic and hypochromic erythrocytes (Urbinati, *et al*. 2006). When excess hemoglobin is present in its non-tetrameric form, it becomes unstable and the α-chain precipitates on membrane structural proteins. The α hemoglobin chain also reacts with oxygen to form peroxyl radicals ($ROO^{\cdot}$), which can lead to significant oxidative damage (Urbinati, *et al*. 2006; Xie, *et al.* 2007; Sassa, 2004). Degradation of excess α-chains promotes autoxidation of hemoglobin to methemoglobin (MetHb) that contains Fe^{3+} instead of Fe^{2+} and thus cannot carry oxygen (Nagababu, et al. 2008). Finally, the dissociation of heme to free heme and iron, followed by their reaction with hydrogen peroxide, generates a cascade of oxidative reactions that destabilize cell membranes (Nagababu, et al. 2008; Graca-Souza, *et al.* 2005). About 365 000 infants are born each year with βthalassemia worldwide and these patients will exhibit many chronic symptoms throughout their lives, including iron over-

load from repeated transfusions (Urbinati, *et al.* 2006), making this disease of significant interest to health science researchers.

HO-1 also has great physiological importance as an antioxidant (Ponka, 1999; Abraham, *et al.* 2008). Several cellular signaling cascades are thought to increase HO-1 expression in response to oxidative stress (Maines, 2005). As well, stress response elements (StREs) within the HO-1 gene promoter have been identified (Poss, et al. 1997). Biliverdin, a product of heme degradation, has been proposed to mediate this StRE activity because it is an antioxidant (Ponka, 1999). Also, since heme has the potential to generate reactive oxygen species (ROS), its removal may be one way in which HO-1 prevents oxidative stress. However, it has recently been suggested that the anti-stress functions of the enzyme are independent of cellular heme catabolism, and that oxidative protection is conferred through a different mechanism (*Sheftel, et al.* 2007). Since HO-1 is upregulated in several cell types in response to oxidative stress, this begs the question of whether HO-1 plays any role in the pathophysiology of β thalassemia, where oxidative damage is incurred through the mechanisms described previously. Our research seeks to clarify whether HO-1 is upregulated in the β thalassemic erythroid cell in response to the destructive effects of α-Hb, metHb, free heme and iron that accumulate due to ineffective hemoglobin synthesis.

The cell culture models for βthalassemia to date have been achieved through the entrapment of heme-containing α-hemoglobin chains in erythrocytes, simulating a βthalassemic phenotype through unbalanced globin levels and partially synthesized hemoglobin (Scott, *et al.* 1990; Szuber, *et al.* 2008). Our model mimics the pathophysiological conditions of βthalassemia by promoting the degradation of β globin mRNA through the mammalian RNAi system (Shi, 2003), using short interfering RNA (siRNA) that has sequence homology to β globin mRNA. siRNA methods have previously been used in MEL cells and mouse models to decrease α and β globin levels (Voon, *et al.* 2008; Xie, *et al.* 2007). This technique should decrease β globin protein expression to simulate β+thalassemia, the heterozygous phenotype, by promoting the unbalanced expression of α and β globin and preventing proper Hb synthesis. Murine erythroleukemic (MEL) cells have been frequently used as a model for studying developing erythrocytes (Sheftel, *et al.* 2007; Voon, *et al.* 2008), and thus we used this cell line in our model of β thalassemia. We measured the expression levels of HO-1, β globin and heme in assessing this model. Decreased levels of β globin were expected due to siRNA-mediated downregulation. Converesely, HO-1 levels were expected as a result of upregulated transcription in response to oxidative stress or due to activation from heme that is not bound in a hemoglobin tetramer, so-called "uncommitted heme". Cellular heme will be measured as it is both an activator and the substrate of HO-1.

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Materials and Methods

Cell culture and Transfection

MEL cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum, to which penicillin (100 units/mL of medium) and streptomycin (100 µg/mL of medium) were added (all from Invitrogen). All experiments were conducted in uninduced MEL cells (control cells) as well as cells treated with 2% DMSO (Me_2SO_2) to promote differentiation and hemoglobinization (Friend, et al. 1971). Cells were plated with $0.5x10^6$ cells per well in 2 mL of DMEM with 10% serum and without antibiotics. 100 pmol of Hbb1 (β-globin) siRNA was transfected into MEL cells, using 5 µl of Lipofectamine™ transfecting reagent (Invitrogen), according to the manufacturer's instructions. DMEM medium without serum or antibiotics was used for the transfection. MEL cells treated with Lipofectamine™ without siRNA served as negative controls. Controls containing 'scramble' or redundant siRNA sequences were omitted from the experiments. Cells were kept in a 37° C incubator until they were harvested.

In an initial experiment, cells were either treated with DMSO 48 hours prior to transfection and harvested after 48 hours, or treated 24 hours after transfection and harvested after 48 hours after DMSO treatment. Also for this experiment only, cells were transfected with either 100pmol or 200pmol of siRNA, using 6 µl of transfecting reagent for the latter amount. In all subsequent experiments, DMSO treatment was carried out six hours after transfection.

In the second experiment, cells were harvested at 48, 72 and 96 hours to check whether the transient effects of siRNA attenuated over time.

The third set of experiments consisted of harvesting at 48 and 96 hours after transfection. The 96-hour cells were re-transfected after 48 hours, at which point DMSO was added to the cells previously treated with DMSO, to maintain a 2% concentration®.

siRNA sequence

The following sequence was used as a short interfering RNA to decrease cellular β globin mRNA levels using the RNAi system. The sequence was obtained from Thermo Scientific Dharmacon .
5'-GGGCAGGCTGCTGGTTGTCTAC-3'

Western blot

Cells were harvested and lysed using Munro lysis buffer, a solution of 10mM Hepes (pH 7.6), 3mM $MgCl_2$, 40 mM KCl, 5% glycerol, and 0.2% NP-40). Protein was determined with a Bradford assay, using a protein assay dye reagent (BioRad), and spectrophotometry absorbance of 540nm. Samples were boiled for 7 minutes prior to loading to disrupt the hemoglobin structure. Protein was separated using a 15% SDS-polyacrylamide gel at 40 mA current for 10 minutes, followed by 60mA for the rest of the separation. Gel was then transferred to a nitrocellulose blotting membrane (BioTrace™) at 200 mA for two hours at 4°C. Prestained molecular weight markers (Fermentas™) were used to estimate the molecular mass of proteins. Membranes were cut into strips according to the positions of β actin, HO-1 and globin protein in order to separately treat sections with their respective primary antibodies, and were washed in primary antibody solutions at 4°C overnight. Rabbit primary antibodies against β-actin and HO-1 (StressGen®) were used in a dilution of 1:5000. Globin rabbit primary antibody was used in a 1:10000 dilution to detect β globin protein (MP Biomedicals, Inc). Membranes were treated for two hours at room temperature with rabbit secondary antibody in a dilution of 1:20,000. The western blot was developed using HyBlot CL™ autoradiography film (Denville Scientific Inc). HO-1 and globin protein levels were normalized according to corresponding β-actin levels in each sample.

Heme measurement

MEL cells were transfected and treated with DMSO as described above. A solution of 0.1M citric acid and 0.1M sodium phosphate in a ratio of 1:2 was combined with hydrogen peroxide (1 µL/mL of solution) and 0.5g of o-phenylenediamine dihydrochloride (Sigma) per 25mL of solution. Experimental cells were harvested after 48 hours, 50 µl of each sample was

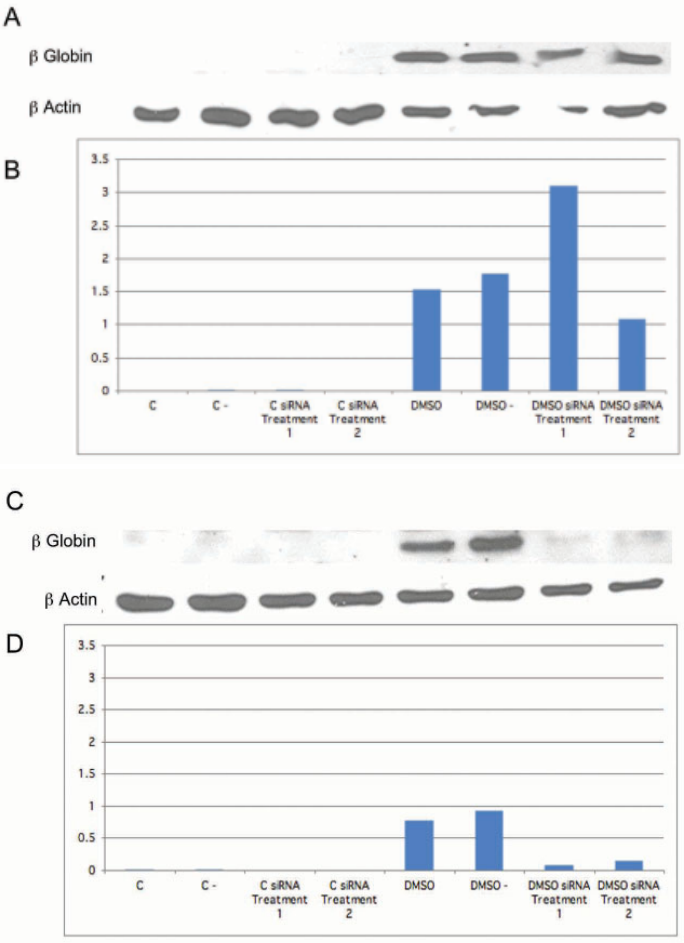

Figure 1: A) Western blot of β globin (Hbb1) and β actin levels in MEL cells treated with DMSO 24 hours prior to transfection with Hbb1 siRNA, harvested 48 hours after transfection. Figure represents the results of one experiment. B) Plot of β globin protein levels in pre-treated MEL cells normalized to β actin expression. C) Western blot of β globin (Hbb1) and β actin levels in MEL cells treated with DMSO 24 hours after transfection with Hbb1 siRNA, harvested 96 hours after transfection. D) Plot of β globin protein levels in MEL cells treated with DMSO 24 hours after transfection, normalized to β actin expression. NOTE: "Treatment 1" refers to 100 pmol of Hbb1-siRNA and 5µl of Lipofectamine™ transfection reagent. "Treatment 2" refers to cells treated with 200 pmol of Hbb1 siRNA and 6 µl of Lipofectamine™ transfection reagent (see methods).

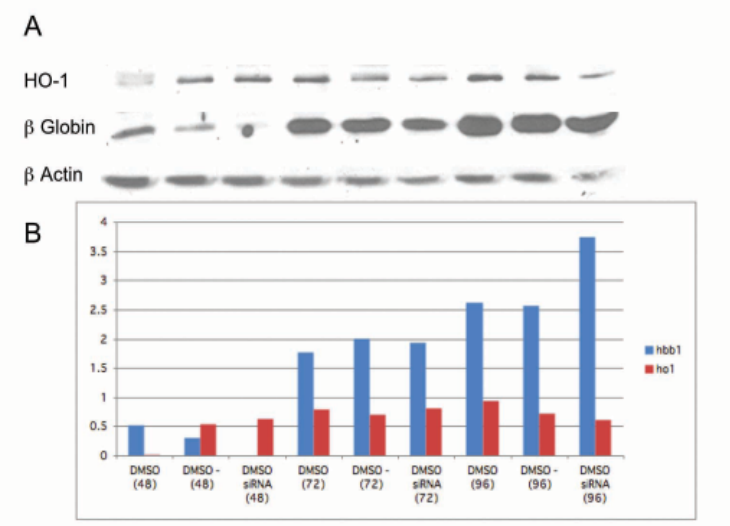

Figure 2: Cells treated with DMSO 6 hours after transfection, and harvested at either 48, 72, or 96 hours. For each time point, cells were either not transfected (DMSO), mock-transfected (DMSO-), or transfected with Hbb1 siRNA (DMSO siRNA). Control cells that were not treated with DMSO are not shown. Figure represents the results of one experiment. A) Western blot of HO-1 and β globin with β actin as a loading reference. Wells correspond to the plots labelled in B). B) Plot comparing β globin with HO-1 levels, normalized to β actin. The Y-axis uses arbitrary units.

collected and lysed by adding 125 µL of the NP-40 Munro Lysis buffer then incubating with cell lysates at 37° C for 5 minutes. Heme reacts with this solution to promote a colour change that is proportional to the level of heme in the lysates. 25 µL of 8 M sulphuric acid was added to the samples to stop the reaction. Absorbance was measured at 492 nm for all lysates.

Results

Protein expression

A single, initial experiment was conducted to determine when differentiation should be induced with DMSO relative to siRNA transfection. Figure 1 shows the results of two separate gels, the first (A, B) represents globin expression in cells pre-treated with DMSO, and the second (C, D) represents globin expression in cells that were induced to differentiate after transfection. Note that in both cases, the variation in globin expression levels in cells that have not been treated with DMSO suggests they have not significantly exceeded the basal level of MEL cell expression. A small reduction in β globin levels was observed in pre-treated cells using 200 pmol of siRNA, but this was not the case for cells treated with 100 pmol of siRNA. If cells are signalled to hemoglobinize before transfected with β globin siRNA, large amounts of the tetrameric globin protein would have been produced before the RNAi system could knock down β globin mRNA levels. However, a large reduction in β globin expression was observed when cells were transfected before DMSO treatment, using 100pmol and 200pmol of siRNA. Subsequent experiments used 100 pmol of siRNA exclusively.

For the following set of experiments, we used a time-course approach to determine the length of time that the transient activity of the siRNA can sustain a reduction in β globin. We also examined levels of HO-1 expression during the period following β globin reduction. Protein levels from cells that were harvested at three different time points were measured using a western blot. Figure 2 depicts cells that were treated with DMSO six hours after transfection. A notable reduction in globin was found in siRNA-treated cells that were harvested after 48 hours, compared with mock-transfected, DMSO-treated cells. In cells transfected after 72 hours and 96 hours, no reduction was observed, and globin levels progressively in-

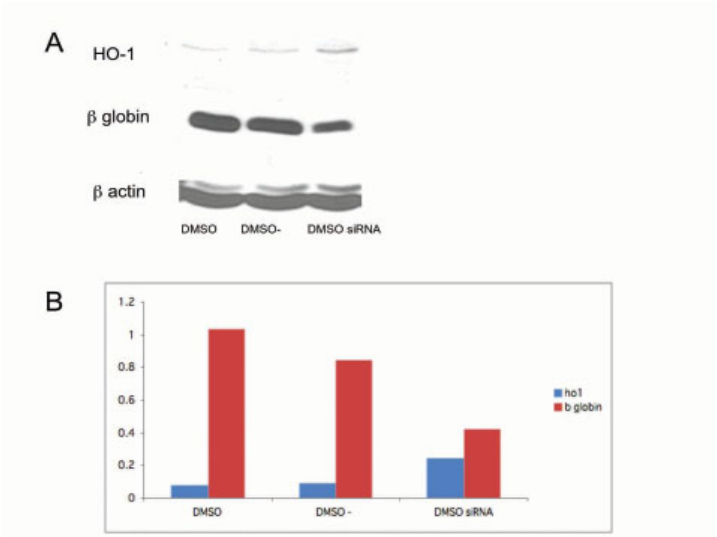

Figure 3: Non-transfected (DMSO), mock-transfected (DMSO-) and transfected cells (DMSO siRNA), harvested after 96 hours when re-transfected 48 hours after initial transfection. Controls untreated with DMSO are not shown. Figure represents the results of a single experiment. A) Western blot of HO-1 and β globin with β actin as a loading reference. B) Plot comparing HO-1 and β globin protein levels, normalized to corresponding β actin. The Y-axis uses arbitrary units.

creased. It appears that the knock-down effects of the siRNA are attenuated after about 48 hours, likely because globin production ensues due to MEL cell differentiation.

The amount of HO-1 did not increase in the same cells that showed a reduction in β globin levels. In Figure 2 B), HO-1 levels are slightly increased in the siRNA-treated cells compared to mock-transfected cells in the 48-hour and 72-hour group. However, no conclusions can be drawn from this experiment regarding the activity of HO-1 in response to β thalassemia-type conditions because globin levels were not sufficiently decreased in the 72-hour and 96-hour cells. These results do suggest that HO-1 levels do not change over time in normally differentiating MEL cells.

In order to maintain decreased levels of β globin mRNA, and to provide time for α-hemoglobin chains to accumulate within the erythrocytes, during the next set of experiments, cells were transfected a second time with siRNA 48 hours after the initial transfection, then harvested after 96 hours. Figure 3 shows a reduction in the globin levels of the transfected cells was observed when compared with mock-transfected cells. A concomitant increase in the levels of HO-1 was observed in transfected cells compared to mock-transfected and non-transfected controls treated with DMSO.

Discussion

Our siRNA approach to developing a β-thalassemic cell model represents a novel tool for investigating the pathology of this acquired disease. We conclude that in order to sustain reduced β-globin protein levels, repeated transfection is required due to the transient knock down effect of siRNA. DMSO-treated MEL cells have a limited lifespan, thus harvesting must take place no later than 96 hours. Although results from all western blots showed decreased globin levels in cells transfected with siRNA, it is necessary to quantify the amounts of α and β globin separately, to clarify whether globin chain synthesis is sufficiently unbalanced to mimic a β-thalassemic phenotype. Further experiments should also include non-specific siRNA sequences to serve as additional controls.

Heme oxygenase-1 expression did not substantially increase in knock-down cells that were harvested 48 hours after transfection with Hbb1 siRNA (Figure 2). However, after 96 hours, cells showed reduced globin levels due to siRNA treatment as well as an observable increase in HO-1 protein levels (Figure 3). Despite the fact that HO-1 protein concentrations only displayed visible increases when they were harvested after 48 hours this does not rule out the possibility that the enzyme's activity may be increasing within the cell in response to pathophysiological changes, even if HO-1 synthesis is not yet being transcriptionally activated.

Heme levels in β globin knock-down cells were distinctly lower than those of DMSO-treated controls (Figure 4). In the MEL cell model, siRNA-mediated reduction of β globin mRNA is theoretically hampering balanced hemoglobin synthesis and may explain the decrease in heme levels. Current understanding of hemoglobin synthesis places suggests heme is a regulator of globin expression (Ponka 1999), but it is possible that the regulatory pathway is more complex than current research would suggest. As well, decreases in β-globin levels may have unknown cis or trans effects on other proteins involved in heme synthesis. One way to further investigate these issues would be to culture transfected cells with $^{59}Fe_2$-transferrin and measure ^{59}Fe incorporation into cells and cellular heme (Sheftel, et al. 2007). Previous results in our laboratory demonstrate that "uncommitted" heme inhibits iron acquisition from transferrin, as well as its incorporation into heme (Ponka, *et al. 1988*). Hence, it is conceivable that heme associated with excess β-globin chains could inhibit iron acquisition from transferrin and consequently, heme biosynthesis. Another possibility is that HO-1 catalyzes the degradation of any heme proteins that are not successfully incorporated into hemoglobin tetramers. This would explain the observed increase in HO-1 expression, which could also be initiated by unincorporated heme.

Finally, it is important to note that the physiological role of HO-1 in the developing erythrocyte is not yet fully understood. Current knowledge suggests that there must be a control mechanism to prevent HO-1 from degrading heme before it reaches a concentration sufficient for hemoglobin synthesis. Therefore, if heme is not degraded by HO-1 during normal cellular differentiation, unbalanced hemoglobin synthesis could result in substantial degradation of unincorporated heme by HO-1.

The role that heme oxygenase plays during erythroid differentiation is still a onditions provides considerable possibilities for future study. Key questions include how HO-1 activity during impaired erythroid development affects the characteristics and lifespan of β-thalassemic red blood cells that enter circulation. HO-1's ability to degrade excess heme and exert

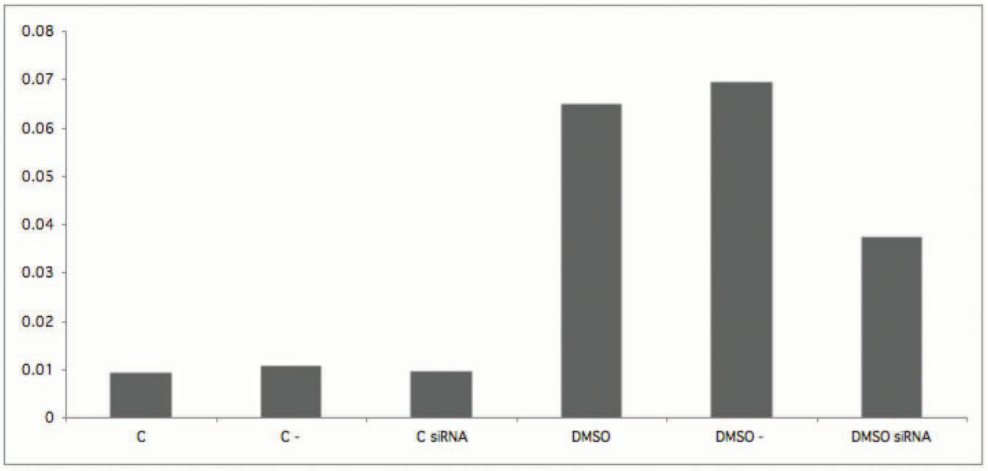

Figure 4: Plot of heme levels in cells harvested 48 hours after transfection, normalized to protein levels of corresponding cell lysates. Non-transfected (DMSO), mock-transfected (DMSO-), or transfected cells (DMSO siRNA), as well as corresponding control cells left untreated with DMSO are shown. The Y-axis depicts arbitrary units of absorbance. Figure represents the results of a single experiment.

a protective effect by reducing free radical generation and oxidative stress is another possible area of investigation. The *Hmox*1 promoter exhibits a $(GT)_n$ repeat polymorphism, which affects the degree to which it is induced in different individuals (Exner et al. 2004). Future research could investigate these polymorphisms in conjunction with the differing severities of β-thalassemic phenotypes that exist across a variety of ethnicities; it is possible that this disease phenotype could be affected by the ability to upregulate heme oxygenase-1.

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