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Hemin: A Possible Cause of Oxidative Stress in Blood Circulation of β-Thalassemia/Hemoglobin E Disease

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A correlation between endogenous hemin and pro-oxidant activity was revealed in serum of β-thalassemia/hemoglobin E disease (β-thal/Hb E), which is the most common prevalent type of thalassemia in Thailand. The technique of low temperature electron spin resonance spectroscopy was used for characterization and quantification of high spin ferric heme, which had been identified as hemin (iron (III)-protoporphyrin IX). Hemin was present at levels ranging from 50 to 280 μM in serum of β-thal/Hb E but not detectable in serum of non-thalassemia. Pro-oxidant activity in serum of β-thal/Hb E was demonstrated by luminol-mediated chemiluminescence, a sensitive method for screening of free radical generation *in vitro*. In the presence of H_2O_2, the chemiluminescence intensity (CL) was about 20 fold enhanced in serum of β-thal/Hb E, indicating its extensive pro-oxidant activity. The CL showed a good correlation with serum hemin, $r = 0.778$ ($p < 0.001$), while the correlations with total serum iron and serum ferritin were 0.260 ($p = 0.259$) and 0.519 ($p = 0.004$), respectively. Our finding suggested that serum hemin readily catalyzed free radical reactions and it may contribute a major pro-oxidant in blood circulation of β-thal/Hb E.

Keywords: ESR; Hemin; Luminol; Pro-oxidant; β-Thalassemia/Hb E

INTRODUCTION

β-Thalassemia is a genetic disorder in which the synthesis of β-hemoglobin chains is abnormally inhibited or absent.[1] Iron overload and depletion of antioxidants in tissues and blood circulation are common in β-thalassemia.[2,3] Oxidative stress, therefore, is thought to be an important mechanism for development of clinical complications in β-thalassemia. Among the complications, atherogenesis-related vascular complications such as pulmonary thromboembolism, cerebral thrombosis and leg ulcers are frequently found.[4–7] Obstructive lesion in the pulmonary artery have been reported in 44% of 43 autopsied thalassemia cases, especially in splenectomized patients.

In addition to vascular endothelial cell injury[8] and platelet abnormality,[9] evidence suggests that oxidative modification of plasma lipoproteins plays a key role in atherogenesis-related vascular alteration in thalassemia.[10,11] Livrea *et al.*[12] have recently showed the relationship between oxidatively modified low density lipoprotein (LDL) and atherogenesis risk in β-thalassemia. The conditions such as rupture of erythrocytes, iron overload and depletion of antioxidant defenses are considered in the promotion of oxidative stress in thalassemic blood circulation.[2,12,13] However, no definitive agent has been identified as a relevant pro-oxidant in the blood circulation of β-thalassemia.

Cannistraro *et al.*[14] have studied iron complexes in serum of β-thalassemia by the technique of electron spin resonance (ESR) spectroscopy. With ESR, both qualitative and quantitative aspects of the transition metals can be examined by virtue of differences in line shape, position in the magnetic field (g values)

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and signal intensity of ESR absorption. Interestingly, the high spin ferric heme ($g = 6$) was detected in the serum of homozygous β-thalassemia which was not present in normal and heterozygous serum. This high spin iron was identified as hemin (iron (III)-protoporphyrin IX). Hemin and other heme compounds are known as potent oxidants that cause lipid peroxidation and free radical generation *in vitro*.[15] Hemin is also a powerful *in vitro* inducer of lipoprotein oxidation, implicated in atherogenesis.[16,17]

Since the amount of hemin is limited in the circulation in normal physiological conditions, the pro-oxidant activity of endogenous hemin has not been reported. In this study, we attempted to investigate the relationship of hemin and pro-oxidant activity in the serum of β-thalassemia/hemoglobin E disease (β-thal/Hb E), which is the most common prevalent type of thalassemia in Thailand.[18] Our finding may suggest a relevant cause of oxidative stress in blood circulation of β-thal/Hb E.

MATERIALS AND METHODS

Chemicals

6-Amino-2,3-dihydro-1,4-pathalazinedione (luminol), hemin chloride (from bovine) and all of other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Study Subjects

Blood samples were collected from 12 healthy volunteers and 32 β-thalassemia/Hb E patients. The patients were recruited from Thalassemia Center, Siriraj Hospital, Mahidol University, Bangkok. They were clinically classified in two groups of 18 non-splenectomized (nsp-thal) and 14 splenectomized (sp-thal). They did not have any blood transfusion for at least 1 month before donating their blood for the studies and were also asked not to take any medications except their daily folic acid supplementation. Characteristics of the subjects are shown in Table I. To determine serum TBARs, serum pro-oxidant activity and plasma α-tocopherol, freshly prepared samples were used. For other parameters, serum samples were stored at −70°C and used within 1 week.

Iron Status

Serum iron levels were measured as recommended by the ICSH.[19] Serum ferritin levels were measured by enzyme immunoassay as previously described.[20]

Lipid Peroxidation

The concentration of thiobarbituric acid reactive substances (TBARs) in serum was measured fluorometrically using a modification of the methods of Asakawa and Matsushita[21] and Uchiyama and Mihara[22] The concentration of TBARs was determined with a spectrofluorometer (Jusco) with 515 nm excitation and 553 nm emission. 1,1,3,3-Tetraethoxypropane was used as the standard.

α-Tocopherol

Levels of α-tocopherol in plasma were determined by using a reverse phase HPLC. The chromatographic system consisted of Waters 715 ULTRA WISP sample processor equipped with an automatic sample injector, a Waters 600E system controller pump and a Waters 470 fluorescence detector. A Nova-Pak C18 (4 mm; 3.9 × 150 mm) column was used in the separation of α-tocopherol. An HPLC grade (100%) methanol was used as the mobile phase. The detector was operated at 295 nm excitation and 370 nm emission.

ESR Measurement

The 300 μl of serum samples were placed in a quartz sample tube (Joel Datum) and then placed in a dewar filled with liquid nitrogen for ESR measurement. ESR spectra were recorded with a X-band ESR (JEOL RX-1) spectrometer equipped with TE011 cavity. The microwave frequency was 9.4 Hz and the power

TABLE I Characteristics of non-thalassemia and β-thalassemia/Hb E subjects

		β-Thalassemia/Hemoglobin E	
	Non-thalassemia	Nsp-thal	Sp-thal
Number of subjects	12	18	14
Age (years)	27.9 ± 7.4	29.9 ± 10.2	28.5 ± 5.7
Sex (M/F)	5/7	7/11	6/8
Hemoglobin (g/dl)	12.5 ± 1.2[a,b]	6.5 ± 0.8[a]	6.1 ± 1.0[b]
Years after splenectomy	–	–	15.7 ± 9.6
Last blood transfusion	–	2 months-5 years	2 months-10 years

Data are presented as mean ± S.D. Significant difference are indicated by different letters at [a,b]$p < 0.001$. Nsp-thal and sp-thal are non-splenectomized and splenectomized thalassemia, respectively.

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was 5 mW. The amplitude of the 100 kHz field modulation was 1.25 mT. The external magnetic field was swept at a scan rate of 37.5 mT/min.

Assay of Pro-oxidant Activity

Pro-oxidant activity in serum of non-thalassemia and β-thal/Hb E was sensitively determined by luminol-mediated chemiluminescence.[23,24] Luminol was dissolved in glycine buffer (pH 12) to 1 mM. The reaction mixture consisted of 10 μl serum and 0.1 mM luminol in 10 mM sodium phosphate buffer pH 7.4. The reaction was started by adding 100 μl of 1 mM H_2O_2 via automatic injector. Chemiluminescence intensity (CL) was determined at 1 min intervals with a Berthold luminometer (Lumat LB 9510/16). The emitted light was measured by a selected high sensitively low noise photomultiplier and expressed in Relative Light Unit (RLU). Its spectrum sensitivity covers the wavelength of 290–630 nM. All of the reactions were performed at room temperature and in a new plastic tube.

Statistic Analysis

Statistic analyses were carried out using Stat View 4.0. The data were analyzed by one way analysis of variance (ANOVA), and Dunn's test used as a post test. The 0.05-level was selected as the minimum level of statistical significance. All of results are presented as the mean ± standard deviation (SD).

RESULTS

ESR spectra of serum of non-thalassemia and β-thal/Hb E at the liquid nitrogen temperature (77 K) are shown in Fig. 1. Signal of Cu^{2+}-ceruloplasmin and Fe^{3+}-transferrin were detected at $g = 2.04$ and 4.3, respectively.[14] In addition to those signals in serum of non-thalassemia, the signal at about $g = 6$ was also detected in serum of β-thal/Hb E. The signal was split into two resolvable g values, $g = 6.09$ and 5.82 which had been characterized as high spin ferric heme embedding in protein matrices.[14,25] The signal intensity was not altered by potent iron chelators, 3-hydroxypyridine-4-one (CP94) nor desferrioxamine. Therefore, the signal should come from heme iron, not from low molecular weight iron which may exist in serum of high iron overloaded patients.

The addition of hemin chloride to serum of non-thalassemia showed ESR signal at $g = 6$ with the same line shape as that of β-thal/Hb E (Fig. 2b). Pre-heating serum at 70°C for 45 min did not alter the signal intensity and line shape of the resonance

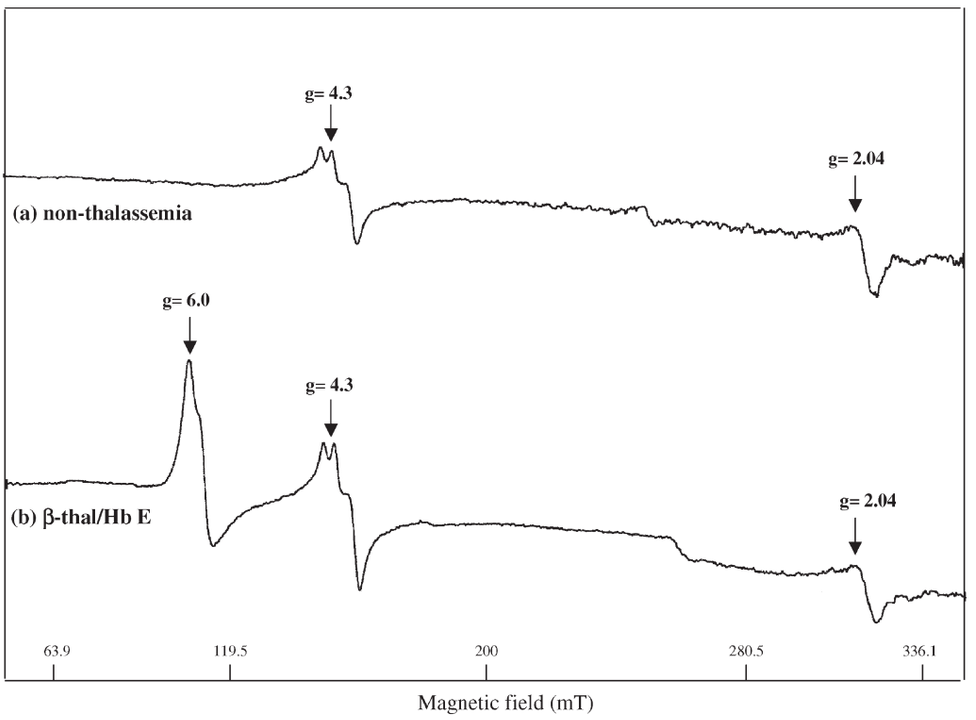

FIGURE 1 ESR spectrum from serum of non-thalassemia (a) and β-thal/HbE (b) recorded at liquid nitrogen temperature (77 K). ESR spectrum were recorded with X-band ESR (JEOL RX-1) spectrometer equipped with TE011 cavity. Microwave frequency was 9.4 Hz and the power was 5 mW. The amplitude of 100 kHz field modulation was 1.25 mT. Magnetic field sweep rate was 37.5 mT/min and time constant 0.3 s.

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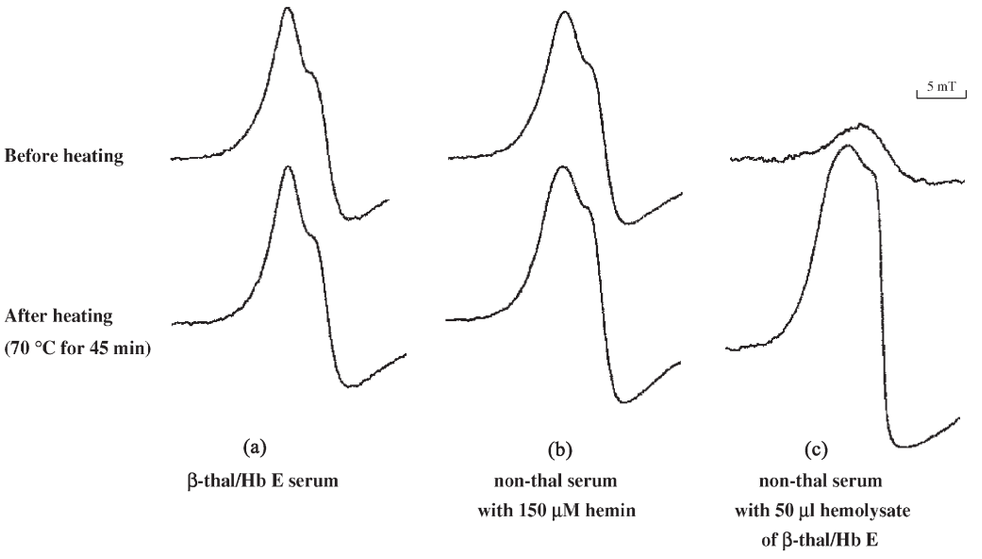

FIGURE 2 ESR signal at $g = 6$ of the serum samples before and after heating: β-thal/Hb E (a), non-thalassemia added with 150 mM hemin (b), and non-thalassemia added with 50 μl hemolysate from β-thal/Hb E (c). The 300 μl of serum samples, before and after heating at 70°C for 45 min, were used for ESR measurement. ESR signal at $g = 6$ were recorded at liquid nitrogen temperature with X-band ESR (JEOL RX-1) spectrometer equipped with TE011 cavity. Microwave frequency was 9.4 Hz and the power was 5 mW. The amplitude of 100 kHz field modulation was 1.25 mT.

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$g = 6$ in serum of β-thal/Hb E or non-thalassemia added with hemin (Fig. 2a,b). On the other hand, ESR intensity was dramatically increased by pre-heating serum of non-thalassemia with hemolysate from β-thal/Hb E (Fig. 2c). This result confirmed that ESR signal at $g = 6$ in serum of β-thal/Hb E did not arise from hemolysis that perhaps occurred during sample preparation.

The ESR signal intensity (peak to trough height) showed linear relationship with known amount of hemin added to serum of non-thalassemia ($r = 0.996$). Therefore, the level of serum hemin could be directly determined by measurement of the ESR signal intensity. The concentrations of serum hemin in nsp-thal and sp-thal subjects are shown in Table II. Serum hemin of both groups varied from low concentration 50 μM to markedly high concentration 280 μM. Although there was no significant difference in levels of serum hemin between nsp-thal and sp-thal, we observed low level of serum hemin (50–120 μM) in the patients with mild clinical severity (Hb > 7.0 g/dl) and low level of lipid peroxidation (serum TBARs 0.06 ± 0.04 nmol/ml). Serum hemin showed a slight but significant correlation with serum TBARs ($r = 0.352$, $p < 0.05$) and plasma α-tocopherol ($r = -0.382$, $p < 0.05$). In addition to serum hemin, parameters for iron status were also determined. Sp-thal showed significant higher iron overload with oxidative stress than that of nsp-thal. There was a good correlation between serum ferritin and TBARs ($r = 0.774$, $p < 0.0001$).

Pro-oxidant activity in serum of non-thalassemia and β-thal/Hb E was determined by luminol-mediated chemiluminescence (Fig. 3). The basal luminescence intensity (in the absence of H_2O_2) was detectable in serum of β-thal/Hb E, indicating the auto-oxidation of luminol by iron and hemin. In the presence of H_2O_2, the CL was dramatically enhanced

TABLE II Levels of serum hemin, iron status and oxidative stress markers of non-thalassemia and β-thalassemia/Hb E subjects

		β-Thalassemia/Hemoglobin E	
	Non-thalassemia	Nsp-thal	Sp-thal
Serum hemin (μM)	Nd	174.3 ± 57.7	195.5 ± 48.2
Iron status			
Total serum iron (μM)	21.3 ± 12.8[a]	24.7 ± 6.6[b]	39.7 ± 7.0[a,b]
% Transferrin saturation	47.6 ± 25.5[c]	73.5 ± 23.0[d]	111.4 ± 12.8[c,d]
Serum ferritin (ng/ml)	57.1 ± 35.8[e]	713.1 ± 601.6[f]	2763.5 ± 2022.9[e,f]
Oxidative stress marker			
Serum TBARs (nmol/ml)	0.17 ± 0.07[g]	0.16 ± 0.11[h]	0.31 ± 0.19[g,h]
Plasma α-tocopherol (μg/ml)	8.90 ± 1.69[i,j]	1.18 ± 1.28[i]	0.63 ± 0.24[j]

Data are presented as mean ± S.D. Significant differences are indicated by different letters at [a,b]$p < 0.001$, [c,e,f,i,j]$p < 0.0001$, [d,g,h]$p < 0.05$. Nsp-thal and sp-thal are non-splenectomized and splenectomized thalassemia, respectively. Nd is not detectable.

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in serum of nsp-thal and sp-thal but not in non-thalassemia, indicating the markedly free radical production in serum of β-thal/Hb E. The CL showed a good correlation with serum hemin, $r = 0.778$ and $p < 0.001$ (Fig. 4), slightly with serum ferritin ($r = 0.519$, $p = 0.0041$) but not with total serum iron ($r = 0.260$, $p = 0.259$). The results suggested that pro-oxidant activity in serum of β-thal/Hb E was mainly contributed by high spin ferric heme or hemin.

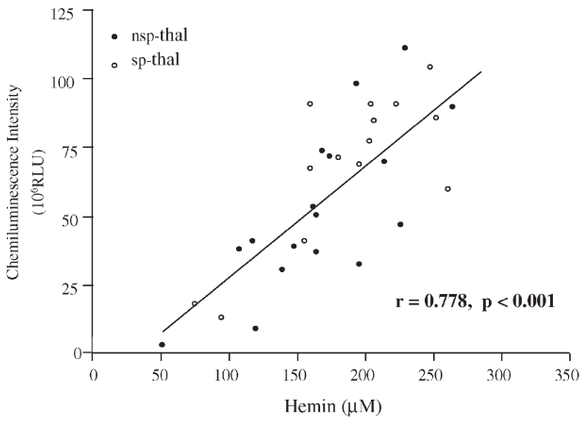

FIGURE 4 Correlation between levels of serum hemin concentration and chemiluminescence intensity induced by H_2O_2. The line was obtained by regression analysis ($r = 0.778$, $p < 0.001$).

DISCUSSION

Hemin (iron (III)-protoporphyrin IX), a denaturative product of hemoglobin, was detected in serum of β-thal/Hb E with micro molar range by using low temperature ESR spectroscopy. ESR signal of hemin ($g = 6$) is split into two resolvable g values, $g = 6.09$ and 5.82, indicating a departure from tetragonal symmetry of heme iron toward rhombic by the protein environment[14] such as hemopexin and albumin.[26]

Hemin may appear in plasma due to intravascular hemolysis, free hemoglobin released into blood plasma is rapidly oxidized to methemoglobin, and subsequently dissociates into hemin and globin.[27] In another pathway, hemin may directly release from red blood cell, without cell lysis. Oxidation of hemoglobin occurring within intact erythrocytes generates a membrane-associated hemin which is readily transferred to plasma constituents.[28] Hemin is bound to plasma hemopexin and albumin and this limits its oxidizing capacity.[29] In normal situations the amount of hemin in circulation is negligible because of the rapid clearance of hemin-bound hemopexin.[30] In β-thalassemia, unstable hemoglobin undergoes extensive oxidation and release of heme moiety. The rate of hemin release is dependent on several factors including the hemoglobin variants, which is high in Hb E disease.[31] It may be speculated that the ability to release hemin by Hb E may play a role in the pathophysiology of β-thal/Hb E.

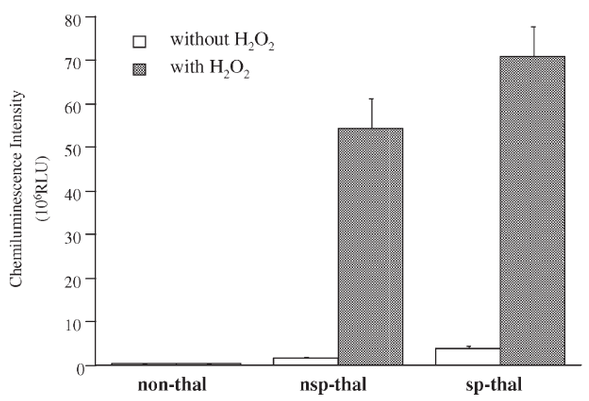

FIGURE 3 Chemiluminescence intensity in serum of non-thalassemia and β-thal/Hb E. The reaction mixture contained 0.1 mM luminol and 100 μl serum in 10 mM sodium phosphate buffer pH 7.4. Chemiluminescence intensity was measured at first 1 min interval after addition of 0.1 mM H_2O_2. Results are expressed in relative light unit (RLU) and presented as mean ± SD of $n = 12$, 18, 14 for non-thalassemia, non-splenectomized (nsp-thal) and splenectomized (sp-thal) thalassemia, respectively.

At micromolar concentrations, hemin has been shown to induce free radical reaction and lipid peroxidation *in vitro*. Cytotoxic effects of hemin were enhanced in the presence of a small amount of hydrogen peroxide and can be prevented by glutathione and vitamin E, indicating the contribution of an oxidative mechanism.[15] Recently, hemin has been reported as a powerful *in vitro* inducer of lipoprotein oxidation, implicated in development of atherosclerosis. Camejo *et al.*[32] have reported that hemin bound to isolated LDL with high affinity and thereby triggers LDL oxidation. LDL oxidized by hemin was shown to be extremely cytotoxic to cultured aortic endothelial cells, supporting involvement of hemin in atherogenesis.[16,17]

We suspected hemin as a potent pro-oxidant in serum of β-thal/Hb E because of its high concentration and its potent pro-oxidant activity *in vitro*. In attempts to study the contribution of serum hemin on oxidative stress, the correlation between serum pro-oxidant activity and hemin was determined by luminol-mediated chemiluminescence. The photo-emissive luminol oxidation is caused by various oxygen-centered radicals, such as superoxide, hydroxyl radical and alkoxyl radical formed by homolytic scission of the hydroperoxide.[23] Although luminol-mediated chemiluminescence lacks specificity for the initial oxygen species responsible, the sensitive method is excellently

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suited for screening for free radical generations *in vitro* and *in situ*.[24]

Our results demonstrated that serum of β-thal/Hb E contained reactive pro-oxidants, which readily react with hydrogen peroxide to produce abundant amounts of reactive oxygen species (ROS). There was a good correlation between hemin concentration and H_2O_2 induced CL in serum of β-thal/Hb E ($r = 0.778$, $p < 0.001$), suggesting the pro-oxidative role of hemin. ESR spectrum indicated that hemin was bound to protein matrices; however, its high concentration readily catalyzed free radical production.

It has been hypothesized that elevated serum ferritin was responsible for oxidative stress and incidence of atherosclerosis. Halle *et al.* have reported that serum ferritin concentration was unlikely to be associated with atherogenesis risk.[33] We found that although serum ferritin concentration showed a good correlation with level of lipid peroxidation ($r = 0.774$, $p < 0.001$), the correlation with CL was low ($r = 0.519$, $p = 0.004$). Therefore, it may be possible that not only ferritin itself but also other iron compounds could be responsible for free radical production and oxidative stress in serum of thalassemic patients.

Here we suggest that hemin may be a factor responsible for oxidative stress in blood circulation of β-thal/Hb E. It may initiate free radical reaction in thalassemic lipoproteins since high concentrations of lipid hydroperoxide were present (our unpublished data). As this is the first report on endogenous hemin and its pro-oxidant activity, we will further investigate the biological and atherogenesis role of hemin in β-thalassemia/Hb E disease.

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