Free Radical Research, March 2006; 40(3): 285–294

A direct and simultaneous detection of zinc protoporphyrin IX, free protoporphyrin IX, and fluorescent heme degradation product in red blood cell hemolysates

QIUYING CHEN[1], & RHODA ELISON HIRSCH[1,2]

[1]*Department of Medicine/Hematology, Albert Einstein College of Medicine, Bronx, NY 10461, USA, and* [2]*Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY 10461, USA*

Accepted by Professor F. Kelly

(Received 19 August 2005)

Abstract

Fluorescence emission of free protoporphyrin IX (PPIX, em. ~ 626 nm), zinc protoporphyrin IX (ZPP, em. ~ 594 nm) and fluorescent heme degradation product (FHDP, em. ~ 466 nm) are identified and simultaneously detected in mouse and human red cell hemolysates, when excited at 365 nm. A novel method is established for comparing relative FHDP, PPIX and ZPP levels in hemolysates without performing red cell porphyrin extractions. The ZPP fluorescence directly measured in hemolysates ($F_{365/594}$) correlates with the ZPP fluorescence obtained from acetone/water extraction ($R^2 = 0.9515$, $P < 0.0001$). The relative total porphyrin (ZPP and PPIX) fluorescence obtained from direct hemolysate fluorescence measurements also correlates with red blood cell total porphyrins determined by ethyl acetate extraction (Piomelli extraction, $R^2 = 0.88$, $P < 0.0001$). These fluorescent species serves as biomarkers for alterations in Hb synthesis and Hb stability.

Keywords: *Porphyrins, fluorescence, heme degradation, oxidation, hemoglobins*

Abbreviations: *Hb, hemoglobin(s); ROS, reactive oxidative species; PPIX, protoporphyrin IX; ZPP, zinc protoporphyrin IX; FHDP, fluorescent heme degradation product; RBC, red blood cell(s); em., emission; ex., excitation*

Introduction

Reactive oxidative species (ROS) are linked to diverse pathophysiologies (e.g. neurodegenerative diseases, diabetes mellitus, end-stage renal disease, cardiovascular, autoimmune diseases and aging). Hemoglobinopathies, arising from unstable hemoglobins and/or ineffective globin chain synthesis, give rise to heme degradation products and ROS. HbE provides the example of the coupling of an unstable Hb with abnormal β-chain synthesis [1]. The instability of sickle cell hemoglobin (HbS) and the cascade of events generating intracellular ROS have been described in detail by Hebbel and others [2–5]. The clinical course of thalassemia arises from an imbalance in globin chain production. In β-thalassemia, β-globin chains are not produced at all (β^0-thalassemia) or in less quantities resulting in the excess highly unstable α-chains that ultimately give rise to ROS [6,7]. Thus, the development of efficient assays to detect ROS and markers of oxidative stress are important for efforts aimed at defining pathophysiological mechanisms.

It has been demonstrated in RBC that non-enzymatic heme degradation is initiated by the autooxidation of hemoglobin (Hb) [8–12] which is normally limited by RBC antioxidants. In the presence of highly unstable Hb, ROS overwhelm the red cell

Correspondence: R. E. Hirsch, Department of Medicine/Hematology, Albert Einstein College of Medicine, Ullmann 925, 1300 Morris Park Avenue, Bronx, NY 10461, USA. Tel: 1 718 430 3604. Fax: 1 718 824 3153. E-mail: rhirsch@aecom.yu.edu

ISSN 1071-5762 print/ISSN 1029-2470 online © 2006 Taylor & Francis
DOI: 10.1080/10715760500522630

antioxidant systems and randomly attack the carbon–methene bridges of the heme moiety tetrapyrrole rings, resulting in fluorescent heme degradation products (FHDPs) and release of free iron [8–12]. Consequently, FHDPs become useful as a sensitive probe to assess the stability of Hb as well as RBC oxidative stress in the peripheral circulation [13–15]. The utility of this assay is demonstrated by significantly elevated levels of FHDP (4–10 fold increases) in transgenic sickle cell mice and thalassemic mice, compared to normal C57 mice [11].

While FHDP serves as a sensitive biomarker of Hb oxidation, elevated RBC porphyrin levels tend to reflect abnormalities in Hb synthesis and/or heme degradation. Free protoporphyrin IX (PPIX) is the intermediate metabolic precursor of the heme molecule. The insertion of ferrous iron into PPIX, catalyzed by the enzyme ferrochelatase, is the final step of heme biosynthesis. A small portion of PPIX normally escapes this process and low levels of free PPIX are observed in erythrocytes. Elevated zinc protoporphyrin (ZPP) levels are observed in iron deficiency anemia and in lead poisoning.

Clinically, the erythrocyte protoporphyrin (both PPIX and ZPP) assay is used to classify RBC disorders resulting from a decrease in heme synthesis (e.g. iron deficiency), chronic diseases, lead toxicity, erythropoietic protoporphyria, disorders of globin production (e.g. thalassemia syndromes), HbC and HbE disorders [16–24]. Indeed, free erythrocyte protoporphyrin levels are a function of three interrelated factors: Iron supply, heme synthesis and levels of erythropoiesis [25,26]. It is well established that erythrocyte protoporphyrin levels increase in diseases associated with heme synthesis disorders. In addition, elevation of erythrocyte protoporphyrin has also been reported in diseases that exhibit a marked increase in erythropoiesis, such as, sickle cell disease [27–29], severe haemolytic anemia and thalassemia major [17,30].

In the present study, the fluorescence emission of PPIX, ZPP and FHDP are detected and identified simultaneously in one fluorescence spectral scan of RBC hemolysates from human and transgenic mice. This one-step method is an extension of the study of Nagababu and Rifkind [8–12] who earlier reported that porphyrin fluorescence was not detected by their oxyHb and red blood cell autooxidation or H_2O_2 oxidation fluorescence assays. A novel and simple direct spectroscopic method is presented that compares the fluorescence emissions of FHDP, PPIX and ZPP in RBC hemolysates. The validation of the method is demonstrated by significant correlations with the established extraction procedure [31,32]. Simultaneous detection of these three parameters provides insight into Hb instability and Hb synthesis, both of which are important for detailing pathophysiological mechanisms of hemoglobinopathies. This direct spectroscopic method is less laborious and, moreover, has the potential for development as a clinical indicator of oxidative stress arising from diseases associated with Hb instability and/or ineffective erythropoiesis.

Materials and methods

Mouse blood was drawn from the tail vein into heparinized capillary tubes using a protocol approved by the animal study committee of the Albert Einstein College of Medicine. The blood was washed three times against isotonic mouse saline (330 m osm) and centrifuged. After removing the supernatant, the packed RBC were lysed in two volumes of distilled water. The lysate was frozen and thawed three times for complete lysis. The concentration of hemolysates was measured by determining the absorbance at 540 nm after conversion to cyanmet Hb by the addition of Drabkin reagent. The samples were diluted with pH 7.35 potassium phosphate buffer, 0.05 M.

PPIX standards (in aliquots of 5 μg) and solid ZPP (not standard, in aliquot 5 mg) were obtained from Frontier Scientifics ((formerly Porphyrin Products), Logan, UT). The PPIX standard (5 μg) was dissolved in 100 μl "Proto–Solv" provided by the company, then further diluted with 10 ml 0.05 M pH 7.35 phosphate buffer. The concentration of PPIX stock solution was calculated from its absorption at 408 nm in 2.7 N HCl (extinction coefficient $\varepsilon = 241$ mM). ZPP was dissolved in 0.1 M NaOH. The concentration of ZPP stock solution was calculated from its absorption at 415 nm in 100% ethanol ($\varepsilon = 115$ mM). Working solutions of PPIX and ZPP were prepared in pH 7.35 potassium phosphate buffer.

Single spectral scan fluorescence assay

A Perkin–Elmer 650–10S spectrofluometer with right angle optics was used to record the fluorescence spectra of FHDP (excitation (ex.) 365 nm, emission (em.) 466 nm), PPIX (ex. 365 nm, em.626 nm) and ZPP (ex. 365 nm, em. 594 nm) in hemolysates (50 μM heme in pH 7.35 50 mM potassium phosphates). For fresh hemolysates without any exogenous oxidants, the fluorescence emissions of FHDP, PPIX and ZPP were measured immediately after preparation and concentration determination of the hemolysate.

Piomelli extraction assay for total porphyrins and ZPP

Hemolysate total porphyrins were extracted by ethyl acetate/acetic acid (4:1) as described by Piomelli [31]. The ratio of ZPP to total porphyrins was obtained from acetone/water (4:1) extraction as described by

Hart and Piomelli [32]. For a direct porphyrin assessment, a 300 μl hemolysate (1.0 g% in 0.9% NaCl) was used for porphyrin extraction instead of 20 μl whole blood as described by Piomelli [31].

Results

Autoxidation and H_2O_2-induced oxidation of mouse hemolysates

The propensity of Hb to oxidize has clinical significance for a variety of hemoglobinopathies. The oxidation of purified Hb or red cell hemolysates generates a variety of by-products and potential ROS. It has been shown that autooxidation of oxyHb results in the production of superoxide anions that dismutate to form H_2O_2 [9]. Thus, in both autooxidation and H_2O_2 oxidation of oxyHb, ferrous and ferric Hb react with H_2O_2 to form oxyferryl Hb, along with the formation of two FHDPs: Compound I exhibits a 321 nm excitation maximum and 465 nm emission maximum, while compound II exhibits an excitation of 460 nm, with emission at 525 nm [7–10]. Compound I has thus far been identified as a dipyrrole derivative, while compound II remains elusive [11].

The fluorescence excitation and emission spectra of FHDP in a HbE mouse [33] (partial mouse α and β globin knockout) hemolysate (in the absence and presence of H_2O_2) are shown in Figure 1(a)–(c). In the absence of H_2O_2, FHDP exhibits excitation peaks at ~321 and ~365 nm (constant emission, 466 nm). The ~365 nm excitation efficiency is 75% of the excitation at ~321 nm, and becomes gradually masked by the increasing intensity of the 321 nm

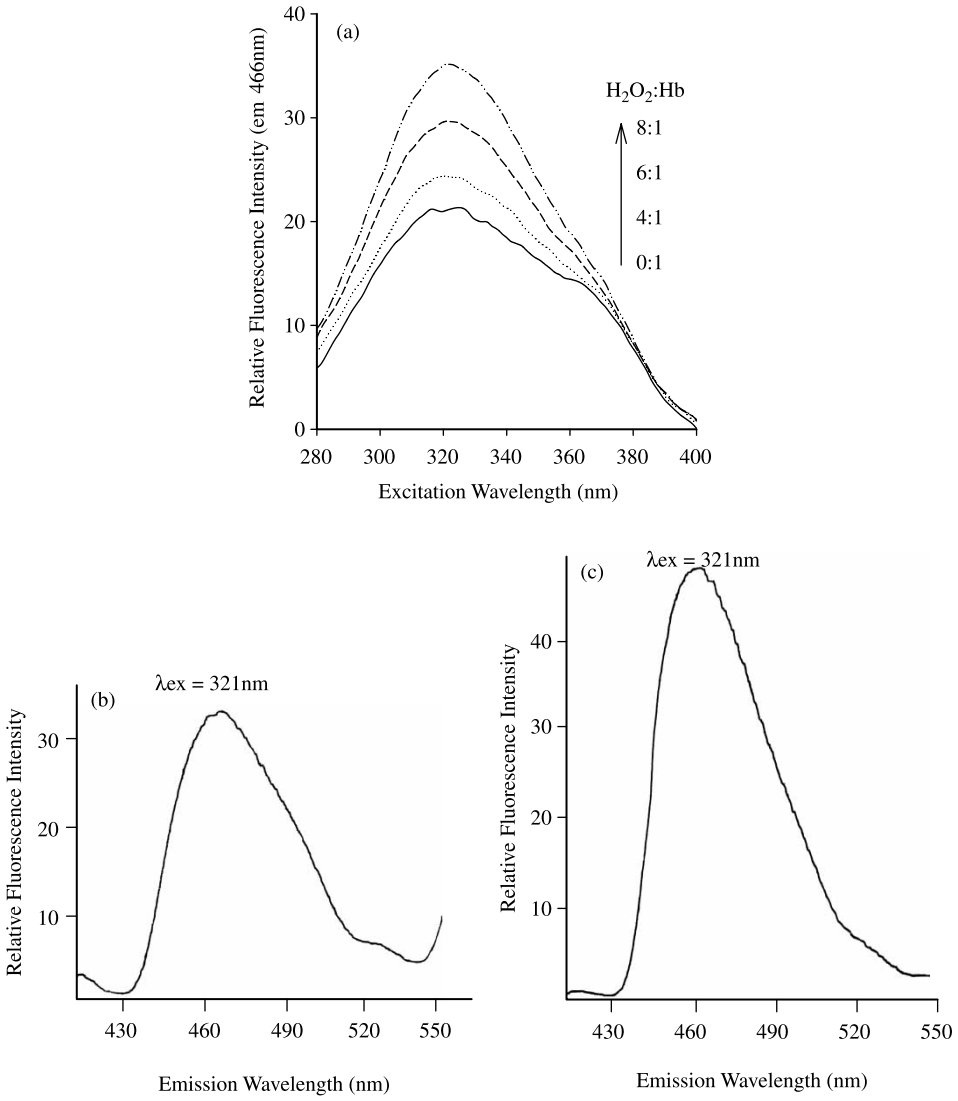

Figure 1. Fluorescence excitation spectra (a) and emission spectra (b), (c) of FHDP in a HbE transgenic mouse hemolysate in the absence and presence of varying concentrations of hydrogen peroxide. (50 μM heme, 50 mM pH 7.35 potassium hemolysates, a 10 nm excitation and emission slit width, sensitivity gain 10). (a) excitation spectra at varying ratios of H_2O_2/Hb, (b) emission spectrum in the absence of H_2O_2, (c) emission spectrum in the presence of 20:1 H_2O_2/Hb. The constant fluorescence emission is at 466 nm for excitation spectra (a) and the excitations are at 321 nm for emission spectra (b), (c).

excitation maximum upon H_2O_2 oxidation (Figure 1(a)). This phenomenon is also seen for the autooxidation and H_2O_2 oxidation of purified hemoglobins.

As mentioned above, both autooxidation and H_2O_2 oxidation generate identical fluorescent products. However, the presence of a large excess of H_2O_2 results in increased heme degradation and more FHDP compared to autooxidation (Figure 1(b) and (c)). Therefore, as more Hb is degraded by H_2O_2, the solution composition consists of less intact Hb and more FHDP molecules. Nevertheless, FHDP are indicators of heme degradation by ROS, since an increase in FHDP is observed in cells [11] with unstable Hb known to generate elevated levels of ROS [2–7].

Fluorescence emissions of PPIX and ZPP are detected in the FHDP fluorescence emission spectral scan

Excitation of a HbE mouse (partial mouse α and β globin knockouts) hemolysate at 365 nm results in a typical fluorescence emission of FHDP (em. ~ 466 nm) and two porphyrin-like fluorescence emissions at ~594 and 626 nm (Figure 2). Although, the 365 nm excitation results in only 75% of the fluorescence intensity of the FHDP as compared to that excited at 321 nm, the advantage of selecting the 365 nm excitation is that the fluorescence emissions of the three fluorescent species are recorded in one spectral scan.

Note that PPIX fluorescence is pH-dependent due to the protonation of four pyrrole nitrogens. The four-protonated form of PPIX (PPIX at acidic solutions) has fluorescence emission at ~600 nm, whereas the two-protonated PPIX (at neutral pH) and non-protonated PPIX (basic pH) have a fluorescence emission at ~626 nm [34]. However, the observed fluorescence emission at 594 nm does not originate from the four-protonated form of PPIX, since at neutral pH, the two-protonated form of PPIX predominates. Indeed, since the signature of ZPP fluorescence emission maximum is at ~594 nm, the ~594 nm emission peak in the hemolysate is assigned to ZPP.

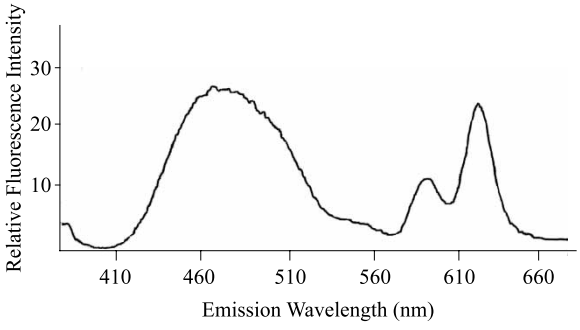

Figure 2. A typical fluorescence excitation at 365 nm results in fluorescence emissions of FHDP (em. 466 nm), ZPP (em. 594 nm) and PPIX (em. 626 nm) in a HbE transgenic mouse hemolysate. 50 μM heme at 50 mM pH 7.35 potassium phosphates.

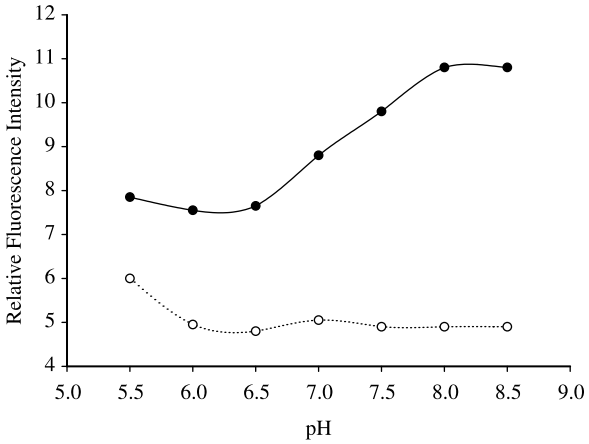

Figure 3. The effect of pH on the 594 nm (open circles, dashed line) and 626 nm (solid circle, solid line) fluorescence emissions in a HbE transgenic mouse hemolysate. 50 μM heme, 50 mM pH 7.35 potassium phosphates, fluorescence excitation at 365 nm.

The presence of both ZPP and PPIX in the hemolysates is corroborated by their differential responses to pH changes. Figure 3 shows the effect of pH on the fluorescence emissions of a hemolysate at 594 and 626 nm. The 626 nm emission, presumably due to PPIX, exhibits a sigmoid shape pH titration curve over the pH range 6–8, consistent with the deprotonation of pyrrole nitrogen in the PPIX tetrapyrrole ring. In contrast, the ~594 nm emission does not respond to pH changes over the pH range 6–8, since all pyrrole nitrogens in ZPP are covalently bound to zinc(II) (i.e. no protonation of the pyrrole nitrogen).

In order to further prove that the 626 nm emission, originating from the hemolysate, is due to PPIX emission, a standard PPIX solution (at pH 7.35 potassium phosphate) is titrated into a HbE transgenic mouse hemolysate. Upon adding PPIX, the ~626 nm emission proportionally increases (Figure 4(a)–(e)), while the emission at 594 nm does not change and eventually becomes masked by the increasing 626 nm emission. Normalized fluorescence excitation spectra were super-imposable before and after PPIX titration (Figure 4(f),(g)). As a control, titration of the hemolysate with a coproporphyrin IX solution results in a fluorescence increase at 615 nm (a ~10 nm-deviation from the intrinsic 626 nm emission of hemolysate (data not shown)), negating its contribution to the 626 nm emission. Therefore, the 626 nm emission observed in the hemolysate prior to PPIX titration originates from erythrocyte PPIX.

Likewise, a standard ZPP solution (at pH 7.35 potassium phosphate) is titrated into a HbE transgenic mouse hemolysate in order to further prove that

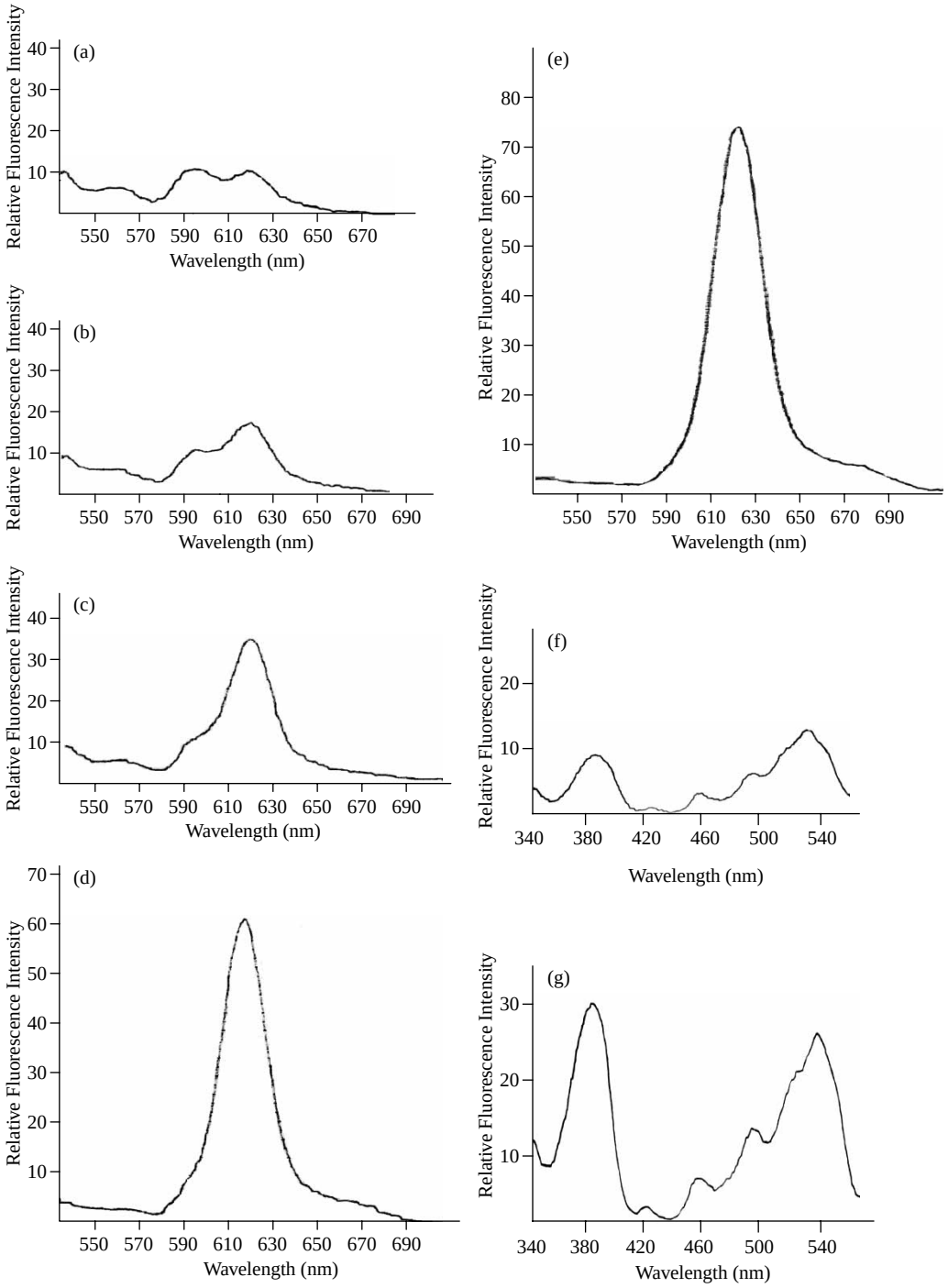

Figure 4. Fluorescence emission (a)–(e) and excitation spectra (f), (g) of a HbE transgenic mouse hemolysate (partial mouse α and β globin knockouts) upon titrating with a PPIX standard solution. 50 μM heme, 50 mM pH 7.35 potassium phosphates. (a) 0 μM PPIX; (b) 0.29 μM PPIX; (c) 0.58 μM PPIX; (d) 1.16 μM PPIX; (e) 1.72 μM PPIX; (f) 0 μM PPIX; (g) 0.58 μM PPIX. Fluorescence excitation is at 365 nm for the emission spectra (a)–(e) and the emission is at 626 nm for the excitation spectra (f), (g).

the ~594 nm emission is due to the presence of ZPP. Upon addition of ZPP, the ~594 nm emission proportionally increases (Figure 5(a–e), (f–g)). Again, normalized fluorescence emission and excitation spectra are super-imposable before and after ZPP titration (Figure 5(f),(g)). It is noteworthy that both ZPP and PPIX have excitation peaks at 360–375 nm that is close to the secondary excitation peak (ex. ~ 365 nm) observed in FHDPs (Figure 1). Therefore, a single excitation at 365 nm detects the presence of FHDP, ZPP and PPIX in hemolysates (Figure 2). In addition, the fluorescence intensities of ZPP and PPIX increase when incubating the hemolysate with H_2O_2 (Figure 6). This is likely due to enhanced heme degradation in

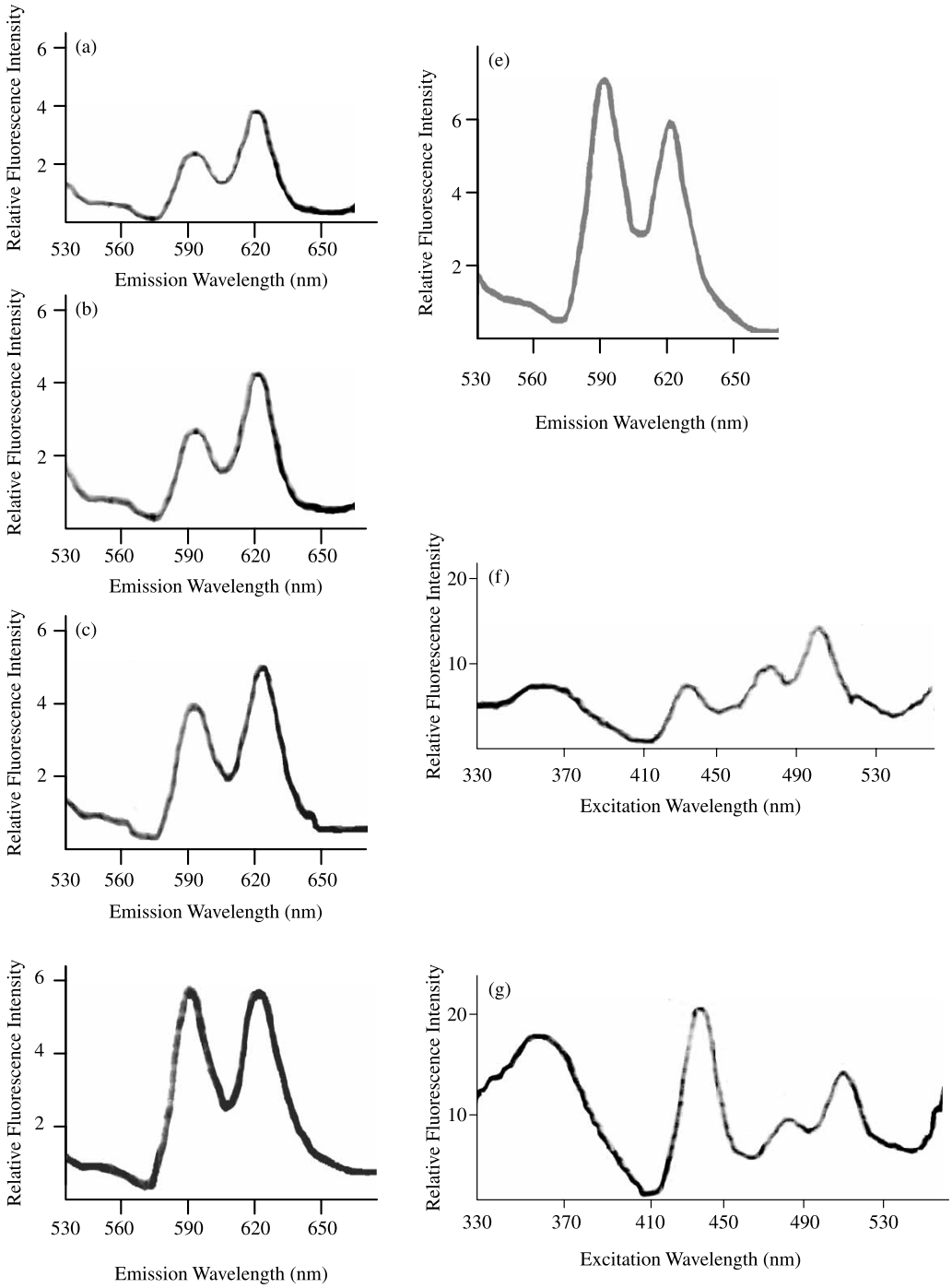

Figure 5. Fluorescence emission (a)–(e) and excitation spectra (f), (g) of a HbE transgenic mouse hemolysate upon titrating with a ZPP solution. 50 μM heme, 50 mM pH 7.35 potassium phosphates. (a) 0 μM ZPP; (b) 0.10 μM ZPP; (c) 0.25 μM ZPP; (d) 0.40 μM ZPP; (e) 0.59μM ZPP; (f) 0 μM ZPP; (g) 0.25 μM ZPP. Fluorescence excitation is at 365 nm for the emission spectra (a)–(e) and the emission is at 594 nm for the excitation spectra (f), (g).

the presence of H_2O_2 and concomitant decreased quenching of porphyrin fluorescence by the heme.

Derivation of the direct spectral determination of the relative total porphyrins and percent ZPP in hemolysates

As shown in Figure 4, PPIX contributions are insignificant at the ZPP primary emission peak (~594 nm), since this peak does not increase upon PPIX titration. In contrast, the 626 nm emission corresponds to the fluorescence of PPIX with a contribution by the secondary ZPP emission at ~626 nm (Figure 5). Consistently, in a separate titration of HbE mouse hemolysate with ZPP and PPIX, the ZPP fluorescence emission ($F_{365/594}$) does not change during PPIX titration (Figure 7). In contrast,

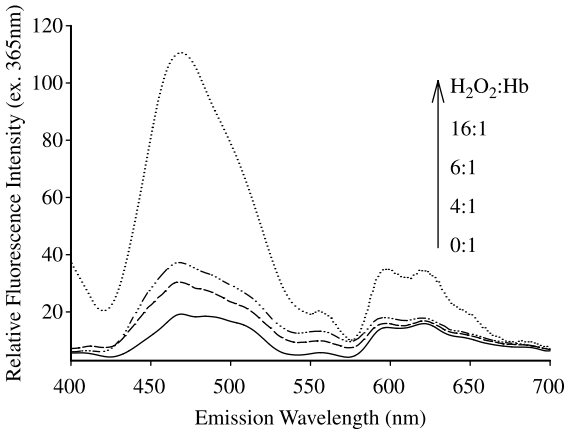

Figure 6. Fluorescence emissions of hemolysate ZPP and PPIX as a function of H_2O_2 concentration. 50 μM heme, 50 mM pH 7.35 potassium phosphates. The hemolysates were derived from a HbE transgenic mouse.

the PPIX fluorescence emission ($F_{365/626}$) increases during ZPP titration (Figure 7). Based on the slopes of the titration curves, the ratio of ZPP fluorescence intensity at the secondary emission (626 nm) and the primary emission (594 nm) can be determined (10.429/51.406 = 0.20). Therefore, while the observed fluorescence intensity at ~594 nm ($F^{\text{hemolysate}}_{365/594}$, Figure 2) is directly measured as hemolysate ZPP fluorescence ($F^{\text{ZPP}}_{365/594}$), the PPIX fluorescence is assessed by the 626 nm emission after deducting the contribution of the ZPP secondary emission at 626 nm, equation (1),

$$F^{\text{PPIX}}_{365/626} = F^{\text{hemolysate}}_{365/626} - F^{\text{ZPP}}_{365/626} \quad (1)$$

where $F^{\text{PPIX}}_{365/626}$ refers to the net PPIX fluorescence in the hemolysate with 365 nm excitation and 626 nm emission; $F^{\text{hemolysate}}_{365/626}$ is the observed hemolysate fluorescence at 626 nm with excitation at 365 nm and $F^{\text{ZPP}}_{365/626}$ is the secondary fluorescence emission of ZPP at ~626 nm (ex. 365 nm). In the case of elevated ZPP, the contribution to the 626 nm may be greater. However, subtraction of the ZPP fluorescence at 626 nm eliminates this contribution and yields a more realistic relative amount.

Substituting $F^{\text{ZPP}}_{365/626}$ in equation (1) with $F^{\text{ZPP}}_{365/594} \times 0.20$ (the ratio between the ZPP secondary fluorescence at 594 nm and primary fluorescence at 626 nm (Figure 7) as described above), the net PPIX fluorescence at ~626 nm can be obtained from equation (2).

$$F^{\text{PPIX}}_{365/626} = F^{\text{hemolysate}}_{365/626} - \left(F^{\text{ZPP}}_{365/594} \times 0.20\right) \quad (2)$$

After obtaining the net PPIX and ZPP fluorescence in the hemolysate, the relative total porphyrin fluorescence ($F^{\text{relative total PPIX}}_{365/626}$) can be calculated. The ratio of the fluorescence quantum yields of ZPP to PPIX in the hemolysates is determined so that the observed ZPP fluorescence can be "converted" to the corresponding PPIX fluorescence. Thus, the total relative porphyrin fluorescence is the sum of the net PPIX fluorescence and the converted ZPP fluorescence.

It is observed that at a 1:1 molar ratio of ZPP and PPIX, the fluorescence emission at ~626 nm is much greater than that at ~594 nm. The fluorescence quantum yield ratio of PPIX and ZPP

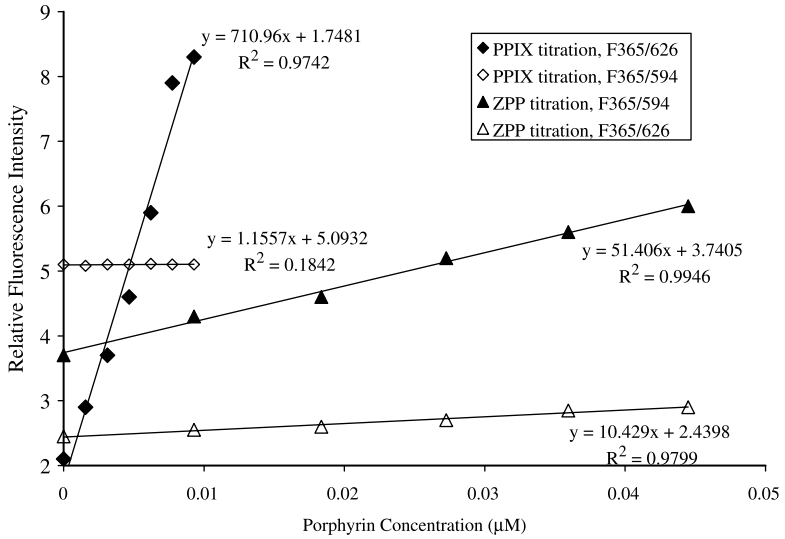

Figure 7. Titration of standard ZPP and PPIX separately into a HbE transgenic mouse hemolysate. 50 μM heme, 50 mM pH 7.35 potassium phosphate. As a control, the ZPP fluorescence intensity (ex. 365 nm, em. 594 nm) as a function of PPIX titration, and the PPIX fluorescence intensity (ex. 365 nm, em. 626 nm) as a function of ZPP titration are also shown. Note that the fluorescence quantum yield difference between ZPP and PPIX is reflected by the differential slopes of the titration curves, with a ratio of PPIX/ZPP = 710.96/51.406 = 13.83.The ratio between the ZPP secondary fluorescence at ~626 nm and the primary fluorescence at ~594 nm can also be obtained as 10.429/51.406 = 0.20.The 594 nm emission does not change during PPIX titration while the 626 nm emission increases upon ZPP titration.

(i.e. $F^{PPIX}_{365/626}/F^{ZPP}_{365/594} = 13.83$) is obtained from the ratio of the slopes of the respective ZPP and PPIX standard titration curves as shown in Figure 7. Based on this ratio, the fluorescence intensity of one molar ZPP at ~594 nm would correspond to 13.83 fold of the fluorescence intensity of one molar PPIX at ~626 nm, should zinc be demetalated from ZPP (yielding PPIX). Therefore, the intensity of ZPP emission at ~594 nm would be equivalent to the ~626 nm PPIX emission according to equation (3),

$$F^{ZPP\rightarrow PPIX}_{365/626} = F^{ZPP}_{365/626} \times 13.83 \quad (3)$$

where $F^{ZPP\rightarrow PPIX}_{365/626}$ refers to the corresponding fluorescence intensity at 626 nm when ZPP is converted to PPIX in the hemolysate; $F^{ZPP}_{365/594}$ refers to the ZPP fluorescence intensity at 594 nm. Thus, the relative total porphyrin fluorescence ($F^{\text{relative total PPIX}}_{365/626}$), obtained as the sum of the net PPIX fluorescence emission at 626 nm, equation (2), and the converted 626 nm emission from ZPP, equation (3), is shown in equation (4). In addition, the %ZPP in the hemolysate can be obtained from equation (5).

$$F^{\text{relative total PPIX}}_{365/626} = \text{equation (2)} + \text{equation (3)} \quad (4)$$

$$\%ZPP = \frac{\text{equation (3)}}{\text{equation (4)}} 100\% \quad (5)$$

Based on equations (2), (4) and (5), a comparison of the relative levels of hemolysate PPIX, total porphyrins, and the ratio of ZPP to total porphyrins can be obtained. Exemplary calculations obtained from a typical fluorescence emission of the hemolysate (Figure 2) are illustrated in Table I. RBC hemolysates from normal human HbA and C57 mice exhibit similar basal fluorescence levels of these three fluorescent species, thereby eliminating the possibility of assay interference by varying levels of RBC components.

Correlation of the two methods: Piomelli extraction and direct hemolysate fluorescence measurement of the relative total porphyrin levels and %ZPP in transgenic mouse hemolysates

The determination of porphyrins by solvent extractions [31] utilizes ethyl acetate/acetic acid (4:1) and hydrochloric acid (HCl) to demetal ZPP. Total erythrocyte porphyrins are extracted in the form of free PPIX. The next step uses acetone/water (4:1) [32] to extract both ZPP and PPIX. The ratio of ZPP to total porphyrin (ZPP/total) is obtained from equation (6) [32]

$$\frac{F_{588}}{F_{617.8}} = 3.3576\frac{[ZPP]}{[T]} + 0.2534 \quad (6)$$

where F_{588} is the fluorescence intensity of ZPP at excitation 420 nm and emission 588 nm; $F_{617.8}$ is the fluorescence intensity at excitation 420 nm and emission 617.8 nm (an isosbestic point where equal amount of ZPP and PPIX exhibit equal fluorescence emission [32]); [ZPP] and [T] are molar concentrations of ZPP and total porphyrins. Thus, the fluorescence contribution of ZPP (F^{ZPP}) to the total porphyrin fluorescence (F^{total}, measured by ethyl acetate/acetic acid extraction) is acquired from equation (7),

$$F_{ZPP} = \frac{(F_{588}/F_{617.8}) - 0.2534}{3.3576} F^{\text{Total}} \quad (7)$$

The relative total porphyrin fluorescence in a variety of different transgenic mouse hemolysates ($n = 12$, including transgenic HbA and HbE mice, C57 mice, β-thalassemia mice) is obtained from equation (4). These relative values correlate with the total porphyrin fluorescence measured by ethyl-acetate extraction.

Table I. Exemplary calculations to determine the hemolysate fluorescence of ZPP, PPIX, relative total porphyrin, and percent ZPP from a typical spectrum shown in Figure 2.

Fluorescence	Calculations	Notes
$F^{ZPP}_{365/594}$	8.5	The net ZPP fluorescence in the hemolysate at 594 nm (directly obtained from the observed emission peak shown in Figure 2).
$F^{hemolysate}_{365/626}$	21.5	The observed hemolysate fluorescence at 626 nm (directly obtained from the emission peak shown in Figure 2).
$F^{ZPP}_{365/626}$	8.5 × 0.20 = 1.7	The ZPP secondary fluorescence emission at 626 nm is 0.2 fold of the primary emission at 594 nm.
$F^{ZPP\rightarrow PPIX}_{365/626}$	8.5 × 13.83 = 117.6	The converted fluorescence at 626 nm is 13.83 fold of ZPP fluorescence at 594 nm, if ZPP converts to PPIX.
$F^{PPIX}_{365/626}$	21.5 − 1.7 = 19.8	The net PPIX fluorescence in the hemolysate is obtained by subtracting the ZPP secondary emission at 626 nm from the observed hemolysate emission at 626 nm.
$F^{\text{relative total PPIX}}_{326/626}$	117.6 + 19.8 = 137.4	Relative total porphyrin fluorescence of the hemolysate obtained from the sum of net PPIX fluorescence and the converted fluorescence from ZPP.
%ZPP	117.6/137.4 = 85.6%	Percent hemolysate ZPP is the ratio of ZPP converted fluorescence to total porphyrin fluorescence.

The correlation ($R^2 = 0.88$) is significant at $P < 0.0001$. In addition, the ZPP fluorescence ($F_{365/594}$) directly measured in the hemolysate correlates with the ZPP fluorescence obtained from ethyl acetate and acetone/water extraction (Equation 7) ($R^2 = 0.95$, $P < 0.0001$). Furthermore, the %ZPP obtained from equation (5) (direct hemolysate fluorescence measurement) correlates with ZPP/T obtained from the acetone/water extraction ($R^2 = 0.90$, $P < 0.0001$). Greater than 85% of erythrocyte porphyrins exist in the form of ZPP in the transgenic HbE mice and C57 mice by the direct measurement. However, in β-thalassemia mice, greater than 99% of erythrocyte porphyrin is ZPP, consistent with those reported in human patients [20–22]. All these data validate the method of the direct determination of relative total erythrocyte porphyrin and ZPP levels in hemolysates. The details and significance of these results will be presented elsewhere (manuscript in preparation).

Discussion

A one-step spectral scan methodology is presented for the purpose of comparing relative levels of ZPP, PPIX and FHDP in hemolysates from different transgenic mice. These fluorescence species are potential biomarkers for alterations in Hb synthesis and/or Hb oxidation and degradation, reflected in the elevated levels of ZPP, PPIX and FHDP in pathogenic transgenic mouse hemolysates (manuscript in preparation). The direct detection of relative erythrocyte PPIX, ZPP and FHDP levels has several advantages: (1) it provides an "intrinsic" erythyrocyte porphyrin fluorescence level with minimal risk to the individual (i.e. venipuncture); (2) it is rapid and efficient as it obtains relative levels of ZPP, PPIX and FHDP in one spectral scan without requiring more laborious extraction procedures.

The origins and correlations of elevated levels of FHDP and porphyrins in pathogenic hemolysates remain complicated. In RBC, superoxide produced from Hb autooxidation undergoes dismutation to produce H_2O_2, initiating a cascade of secondary oxidative reactions, which involves heme degradation [10]. Furthermore, superoxide has been shown to react with protoporphyrins to produce FHDP [9,10]. We observed that the fluorescence of FHDP in the hemolysate shows a time and temperature dependent increase in intensity, especially in diluted hemolysate solutions, indicating heme degrades in the hemolysate. In contrast, the levels of ZPP and PPIX levels are relatively stable. The fact that pathogenic transgenic mice (such as sickle cell mice, β-thalassemia and hemoglobin E diseases) exhibit elevated levels of FHDP, ZPP and PPIX (manuscript in preparation) provides evidence that FHDP forms not only *in vitro* but also *in vivo*.

What are the consequences of elevated red blood cell FHDP and porphyrin levels? The FHDP may accumulate on the cell membrane or bind to the Hb, altering membrane properties, red cell deformability and Hb function [11]. It has also been shown that PPIX induces photodynamic damage on RBC membranes by photooxidation of membrane protein amino acid residues and subsequent cross-linking of these proteins [35,36]. *In vitro* spectrophotometric studies reveal that PPIX interacts with Hb, forming ground state complexes and potentiates the peroxidase activities of haemoglobin and myoglobin [37,38]. In addition, the pro-oxidant effects of PPIX have been demonstrated by an *in vivo* administration into a rat model resulting in a rapid induction of superoxide dismutase [39]. The exact mechanism of porphyrin damage is elusive because controversy remains as to the association site of PPIX and ZPP inside the red cells that could include the Hb heme pocket, non-heme pocket sites, and the RBC membrane [40,41].

In summary, ZPP, PPIX and FHDP are important biomarkers of the complex pathophysiology of different hemoglobinopathies. A direct spectral assessment of their relative levels in RBC hemolysates has immediate research application and also clinical potential in the facilitation of the screening and evaluation of oxidative stress, and the status of heme and globin synthesis.

Acknowledgements

We wish to thank Dr Mary E. Fabry, Director of the sickle cell transgenic mouse facility and Sandra M. Suzuka for breeding the mice, and for their helpful discussions; Dr Ronald L. Nagel for his support and encouragement; and Mr Joseph Canterino for technical assistance with the mice. This work has been supported in part by the National Institutes of Health R21 DK 06423; NIH Bronx Comprehensive Sickle Cell Center/Sickle Cell Scholar Award (U54 HL070944), and the American Heart Association, Heritage Affiliate Grant-in-Aid No. 0256390T.

References

[1] Chiu DT, van den Berg J, Kuypers FA, Hung IJ, Wei JS, Liu TZ. Correlation of membrane lipid peroxidation with oxidation of hemoglobin variants: Possibly related to the rates of hemin release. Free Radic Biol Med 1996;21:89–95.

[2] Hebbel RP. Auto-oxidation and a membrane-associated "Fenton reagent": A possible explanation for development of membrane lesions in sickle erythrocytes. Clin Haematol 1985;14:129–140.

[3] Hebbel RP. The sickle erythrocyte in double jeopardy: Autoxidation and iron decompartmentalization. Semin Hematol 1990;27:51–69.

[4] Browne P, Shalev O, Hebbel RP. The molecular pathobiology of cell membrane iron: The sickle red cell as a model. Free Radic Biol Med 1998;24:1040–1048.

[5] Sheng K, Shariff M, Hebbel RP. Comparative oxidation of hemoglobins A and S. Blood 1998;91:3467–3470.
[6] Chan AC, Chow CK, Chiu D. Interaction of antioxidants and their implication in genetic anemia. Proc Soc Exp Biol Med 1999;222:274–282.
[7] Shinar E, Rachmilewitz EA. Oxidative denaturation of red blood cells in thalassemia. Semin Hematol 1990;27:70–82.
[8] Nagababu E, Rifkind JM. Formation of fluorescent heme degradation products during the oxidation of hemoglobin by hydrogen peroxide. Biochem Biophys Res Commun 1998;247:592–596.
[9] Nagababu E, Rifkind JM. Heme degradation during autoxidation of oxyhemoglobin. Biochem Biophys Res Commun 2000;273:839–845.
[10] Nagababu E, Rifkind JM. Reaction of hydrogen peroxide with ferrylhemoglobin: Superoxide production and heme degradation. Biochemistry 2000;39:12503–12511.
[11] Nagababu E, Rifkind JM. Heme degradation by reactive oxygen species. Antioxid Redox Signal 2004;6:967–978.
[12] Nagababu E, Chrest FJ, Rifkind JM. The origin of red cell fluorescence caused by hydrogen peroxide treatment. Biochim Biophys Acta 2003;1620:211–217.
[13] Nagababu E, Ramasamy S, Rifkind JM, Jia Y, Alayash AI. Site-specific cross-linking of human and bovine hemoglobins differentially alters oxygen binding and redox side reactions producing rhombic heme and heme degradation. Biochemistry 2002;41:7407–7415.
[14] Jia Y, Alayash AI. Stopped-flow fluorescence method for the detection of heme degradation products in solutions of chemically modified hemoglobins and peroxide. Anal Biochem 2002;308:186–188.
[15] Jia Y, Wood F, Menu P, Faivre B, Caron A, Alayash AI. Oxygen binding and oxidation reactions of human hemoglobin conjugated tocarboxylate dextran. Biochim Biophys Acta 2004;1672:164–173.
[16] Marsh Jr, WL, Nelson DP, Koenig HM. Free erythrocyte protoporphyrin (FEP) II. The FEP test is clinically useful in classifying microcytic RBC disorders in adults. Am J Clin Pathol 1983;79:661–666.
[17] Koenig HM, Lightsey Jr, AL, Schanberger JE. The micromeasurement of free erythrocyte protoporphyrin as a means of differentiating alpha thalassemia trait from iron deficiency anemia. J Pediatr 1975;86:539–541.
[18] Lyberatos C, Chalevelakis G, Platis A, Stathakis N, Panani A, Gardikas C. Erythrocyte content of free protoporphyrin in thalassaemic syndromes. Acta Haematol 1972;47:164–167.
[19] Meloni T, Gallisai D, Demontis M, Erre S. Free erythrocyte porphyrin (FEP) in the diagnosis of beta-thalassaemia trait and iron deficiency anaemia. Haematologica 1982;67:341–348.
[20] Graham EA, Felgenhauer J, Detter JC, Labbe RF. Elevated zinc protoporphyrin associated with thalassemia trait and hemoglobin E. J Pediatr 1996;129:105–110.
[21] Pootrakul P, Wattanasaree J, Anuwatanakulchai M, Wasi P. Increased red blood cell protoporphyrin in thalassemia: A result of relative iron deficiency. Am J Clin Pathol 1984;82:289–293.
[22] Tillyer ML, Tillyer CR. Zinc protoporphyrin assays in patients with alpha and beta thalassaemia trait. J Clin Pathol 1994;47:205–208.
[23] Brown RG. Determining the cause of anemia. General approach, with emphasis on microcytic hypochromic anemias. Postgrad Med 1991;89(161–164):167–170.
[24] Mei Z, Parvanta I, Cogswell ME, Gunter EW, Grummer-Strawn LM. Erythrocyte protoporphyrin or hemoglobin: Which is a better screening test for iron deficiency in children and women? Am J Clin Nutr 2003;77:1229–1233.
[25] Labbe RF, Finch CA, Smith NJ, Doan RN, Sood SK, Madan N. Erythrocyte protoporphyrin/heme ratio in the assessment of iron status. Clin Chem 1979;25:87–92.
[26] Langer EE, Haining RG, Labbe RF, Jacobs P, Crosby EF, Finch CA. Erythrocyte protoporphyrin. Blood 1972; 40:112–128.
[27] Hirsch RE, Pulakhandam UR, Billett HH, Nagel RL. Blood zinc protoporphyrin is elevated only in sickle cell patients with low fetal hemoglobin. Am J Hematol 1991;36:147–149.
[28] Vichinsky E, Kleman K, Embury S, Lubin B. The diagnosis of iron deficiency anemia in sickle cell disease. Blood 1981;58:963–968.
[29] Yip R. Erythrocyte protoporphyrin in sickle cell disease. J Pediatr 1980;97:163.
[30] Stockman JA, 3rd, Weiner LS, Simon GE, Stuart MJ, Oski FA. The measurement of free erythrocyte porphyrin (FEP) as a simple means of distinguishing iron deficiency from beta-thalassemia trait in subjects with microcytosis. J Lab Clin Med 1975;85:113–119.
[31] Piomelli S. Free erythrocyte porphyrins in the detection of undue absorption of Pb and of Fe deficiency. Clin Chem 1977;23(2 pt. 1):264–269.
[32] Hart D, Piomelli S. Simultaneous quantitation of zinc protoporphyrin and free protoporphyrin in erythrocytes by acetone extraction. Clin Chem 1981;27:220–222.
[33] Chen Q, Bouhassira EE, Besse A, Suzuka SM, Fabry ME, Nagel RL, Hirsch RE. Generation of transgenic mice expressing human hemoglobin E. Blood Cells Mol Dis 2004;33:303–307.
[34] Lamola AA. Fluorescence study of porphyrin transport and clearance. Acta Dermatovener (Stockholm) Suppl 1982;100:57–66.
[35] Dubbleman TM, de Goeij AF, van Steveninck J. Photodynamic effects of protoporphyrin on human erthrocytes. Nature of the cross-linking of membrane proteins. Biochim Biophy Acta 1978;511:141–151.
[36] Dubbleman TM. Photoporphyrin-induced photodynamic effects on band 3 protein of human erythrocyte membranes. Biochim Biophys Acta 1981;649:310–316.
[37] Sil S, Bose T, Roy D, Chakraborti AS. Protoporphyrin IX-induced structural and functional changes in human red blood cells, haemoglobin and myoglobin. J Biosci 2004;29:281–291.
[38] Sil S, Chakraborti AS. Comparative studies on the interaction of protoporphyrin with hemoglobin and myoglobin. Indian J Biochem Biophys 1996;33:285–291.
[39] Afonso S, Vanore G, Batlle A. Protoporphyrin IX and oxidative stress. Free Radic Res 1999;31:161–170.
[40] Hirsch RE, Lin MJ, Park CM. Interaction of zinc protoporphyrin with intact oxyhemoglobin. Biochemistry 1989;28:1851–1855.
[41] Hirsch RE. Porphyrins and hemoglobin. Semin Hematol 1989;26:47–53.