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ผลงานจากโครงการ

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1. Jirasomprasert T, **Morales NP,** Limenta LMG, Sirijaroonwong S, Yamanont P, Wilairat P, Fucharoen S, Chantharaksri U. Pharmaco/ferrokinetic-related pro-oxidant activity of deferiprone in β-thalassemia. Free Radical Research, **2009;** 43 (5):485-491.

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1. **Morales NP,** Jirasomprasert T, Yamanont P, Chunephisal P, Chaivichit P, Chantharaksri U. Fucharoen S. ESR spin trapping/Labeling for evaluation of iron chelator toxicity and oxidative stress in β-thalassemia. Abstract for oral presentation. A Joint Conference of the 13th In vivo ESR/EPR Spectroscopy & Imaging and the 10th International EPR Spin Trapping/Spin Labeling: Biomedical Redox Navigation, 28-30 September 2008, JAL Resort Sea Hawk Hotel Fukuoka, Kyushu, Japan.

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การจัดสัมมนา

1. Redox imaging of animal model with OMRI and innovative research center of medical redox navigation in Kyushu University. Prof. Hideo Utsumi, January 24th, 2008. K102 Chalermprakiate Building, Faculty of Science, Mahidol University.

การเชื่อมโยงทางวิชาการกับนักวิชาการอื่นๆทั้งในและต่างประเทศ

1. Professor Hideo Utsumi: Department of Bio-function Science, Faculty of Pharmaceutical Science and Innovation Center for Medical Redox Navigation, Kyushu University, Fukuoka, Japan ในความร่วมมือการวิจัยศึกษาปฏิกิริยาอนุมูลอิสระในหนูภาวะเหล็กเกินโดย in vivo ESR spectroscopy และ OMRI

2. ศ. นพ. สุทัศน์ ฟู่เจริญ: โครงการวิจัยธาลัสซีเมีย มหาวิทยาลัยมหิดล ในงานวิจัยเรื่องการศึกษาประสิทธิภาพและความเป็นพิษของยาขับเหล็ก deferiprone ในผู้ป่วยธาลัสซีเมีย

3. องค์การเภสัชกรรม สนับสนุนยาขับเหล็กชนิดรับประทาน deferiprone (GPO-L-ONE) และสารมาตรฐาน defriprone

ภาคผนวก

เอกสารที่ 1. Jirasomprasert T, **Morales NP,** Limenta LMG, Sirijaroonwong S, Yamanont P, Wilairat P, Fucharoen S, Chantharaksri U. Pharmaco/ferrokinetic-related pro-oxidant activity of deferiprone in β-thalassemia. Free Radical Research, **2009;** 43 (5):485-491.

เอกสารที่ 2. Chunephisal P, Triampo D, Yamanont P, **Morales NP.** ESR spin labeling studies on the site of iron-induced free radical reaction in low density lipoprotein. Thai Journal of Pharmacology, **2009;** 31: 104-106. Proceeding in 31st Pharmacological and Therapeutic Society of Thailand Meeting, 18-20 March 2009, Khon-Khen University, Thailand.

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Pharmaco/ferrokinetic-related pro-oxidant activity of deferiprone in β-thalassemia

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Abstract

The potential of free radical formation in serum of β-thalassemia/Hb E patients receiving a single oral dose of 25 mg/kg body weight of deferiprone, a bidentate orally active iron chelator, was evaluated using EPR/spin trapping technique. In the presence of ascorbic acid and *tert*-butylhydroperoxide, EPR signals of ascorbyl radical ($a_H = 0.18$ mT) and DMPO-carbon centred adduct ($a_H = 2.37$ mT, $a_N = 1.65$ mT) were detected. Shortly after deferiprone administration, EPR signal intensities decreased concomitant with an increase in serum levels of deferiprone. Unfortunately, enhanced EPR signal intensities were observed at 300 min after dosing in patients with serum molar ratio of deferiprone to iron less than 3, suggesting the formation of incomplete iron-deferiprone complexes and consequently free radical formation. To avoid adverse effects of deferiprone, a dosage regimen should be designed according to iron status of the patients and aimed at maintaining an adequate ratio of serum chelator-to-iron concentration.

Keywords: *Ascorbyl radical, beta-thalassemia, deferiprone, EPR, pro-oxidant, spin trapping*

Introduction

Iron-induced oxidative damage is believed to be one of the major causes of complications in β-thalassemia, an inherited disorder of β-globin chain synthesis, and other forms of iron overload syndromes [1]. Typically, β-thalassemia patients have signs and symptoms related to haemolytic anaemia and ineffective erythropoiesis. As a result of periodic or chronic blood transfusion and increased gastrointestinal iron uptake, patients almost always develop iron overload [2]. The excess iron accumulates and induces a free radical-mediated reaction leading to damage of various tissues and organs including the heart, liver, spleen, kidney and endocrine glands. Without sufficient chelation therapy, patients with β-thalassemia develop fatal iron-related complications, such as hepatic fibrosis and cirrhosis, multiple endocrinopathies (diabetes mellitus, hypogonadism, hypoparathyroidism, hypothyroidism), immunological dysfunction, growth and bone abnormalities, cardiac diseases (congestive heart failure, arrhythmia) and pulmonary dysfunction, often leading to death in the second or third decade of life [3].

Deferiprone (1, 2 dimethyl-3-hydroxypyrid-4-one, also known as L1, MW 139) is an orally active (and inexpensive) iron chelator that has been available for clinical use since 1995 [4]. Being a bidentate chelator, deferiprone forms a strong, water-soluble 3:1 complex with Fe^{3+} ion, with binding constant of 37, which is markedly higher than that of the hexadentate

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iron chelator, deferioxamine [5]. Another advantage of this drug over deferioxamine is that it penetrates cell membrane readily and therefore is effective in removing low molecular weight or labile iron that promotes production of reactive oxygen species from both intra- and extra-cellular pools [5,6]. Moreover, deferiprone has been shown to be more effective than deferioxamine in chelating cardiac iron and thus patients on deferiprone therapy have a remarkably lower prevalence of cardiac disease and cardiac death compared with those chelated with deferoxamine alone [3,7].

However, deferiprone has a limited use as a second line drug or in combination with deferoxamine because of its serious adverse effects including transient agranulocytosis (0.6%), neutropenia (6%) and arthropathy (15%) [8]. The causes of deferiprone-induced toxicity are not known, but those adverse effects are reversible, dose-dependent and likely to occur in severe cases [5]. It has been suggested that both arthropathy and agranulocytosis are associated with the presence of the incomplete formation of deferiprone-iron complexes. At low deferiprone concentration, formation of incomplete 1:1 and 2:1 chelator-iron complexes are formed and the unoccupied coordination sites of these complexes catalyse formation of hydroxyl radical or other reactive oxygen species [9]. Recently, Devanur et al. [10] demonstrated that a 2:1 deferiprone-iron complex accelerates hydroxyl radical production in the presence of ascorbate and hydrogen peroxide in aqueous solution. On the other hand, the fully coordinated deferiprone-iron 3:1 complex has been shown to have antioxidant activity.

In order to monitor toxic side-effects that may have been induced by incomplete deferiprone iron complexes in iron overload patients, electron paramagnetic resonance (EPR) spectroscopy using 5,5-dimethyl-l-pyrroline-*N*-oxide (DMPO) as a spin trapping agent was employed to evaluate free radical formation in sera of β-thalassemia/haemoglobin E (β-thal/Hb E) subjects receiving a single oral dose of 25 mg/kg body weight of deferiprone. Concentration-time profiles of deferiprone, deferiprone-chelated iron and free radical formation were determined, demonstrating for the first time pro-oxidant and antioxidant activities of deferiprone in sera of such patients.

Materials and methods

Subjects

Twenty-one β-thal/Hb E patients were enrolled in the study. Based on haemoglobin level, age at disease presentation, age at first transfusion, frequency of transfusion, degree of hepato-splenomegaly and growth retardation [11], 11 patients were categorized as mild–moderate and 10 patients as severe. None of the patients had received blood transfusion in the month prior to blood sampling or had taken any medication except their daily folic acid supplementation. The study protocol was approved by the Ethics Committee of Ramathibodi Hospital, Mahidol University, Thailand, and all subjects gave written informed consent before participation.

Study design

After overnight fasting, a single oral dose of 25 mg/kg body weight of deferiprone (Ferriprox™, Apotex Inc, Toronto, Ontario, Canada, Lot no. GY4120) was administered followed by 200 ml of drinking water. A standard meal was given 2 h after drug administration. Five millilitres of venous blood samples were collected at pre-dosing and at 15, 30, 45, 60, 90, 120, 180, 240, 300, 360 and 480 min after dosing. Blood samples were collected in siliconized Venojects tubes (Terumo EuropeN.V., Leuven, Belgium) and left for 45 min at room temperature before centrifugation twice at 1700 *g* for 10 min at 4°C and the supernatants were stored at −20°C until analysis (2–4 weeks).

Quantitative analysis of non-glucuronide conjugated form of deferiprone [12]

A 500 µl aliquot of serum sample was deproteinized by centrifuging at 2300 g for 30 min using a Amicon® Centrifree micropartition device (Mw 30 000 cut-off; Millipore, Bedford, MA) and a 20 µl aliquot of the filtrate was used for the determination of the non-glucuronide form of deferiprone employing reversed-phase high-performance liquid chromatography (HPLC). Separation was performed under isocratic conditions on an Eclipse® XDB-C18 column (5 µm, 150 × 4.6 mm i.d.; Agilent Technologies, Waldbornn, Germany) with a guard column in Waters 2695 separations module with autosampler (Waters, Milford, MA). Eluent was monitored at 280 nm by a Waters 2487 dual absorbance detector (Waters, Milford, MA). The mobile phase consisted of 10 mM sodium dihydrogen phosphate, pH 3 (adjusted with phosphoric acid) containing 2 mM ethylenediaminetetraacetate (EDTA) and methanol at 93:7 (v/v). The flow rate was 1.0 ml/min. Peak areas were integrated using Millenium 3.2 software (Waters, Milford, MA).

Quantitative analysis of non-transferrin bound iron (NTBI) and serum deferiprone-chelated iron [13]

Serum samples were mixed with 0.2 M nitrilotriacetate (NTA; Sigma, St. Louis, MO) and allowed to stand at room temperature for 30 min. The solutions were subsequently ultrafiltered through an Amicon® microcon YM-30 filter to separate the resulting Fe (III)-NTA complex from transferrin. Filtrated serum solutions were diluted 1:1 (v/v) with 0.5 M HEPES

buffer (Fluka, Buchs, Switzerland). The 50 µl of 150 mM thioglycolic acid (TGA; Fluka, Buchs, Switzerland) and 50 mM bathophenanthrolinedisulphonic acid (BPT; Sigma, St. Louis, MO) were subsequently added into the solution for reduction of Fe (III) to Fe (II) and colourimetric measurement of the Fe (II)-BPT complex. Solutions were then left for 90 min at room temperature in order to allow formation of the coloured complex to reach equilibrium before measurement of absorbance at 537 nm in a UV-visible spectrophotometer (GBC Cintra 10e, Melbourne, Australia).

Detection of free radical formation in serum by ESR spin trapping

Evaluation of potential free radical formation in serum samples employed *tert*-butylhydroperoxide (*t*-BuOOH) and ascorbic acid to induce a free radical reaction. Free radicals produced were detected by EPR spin trapping technique utilizing 5,5-dimethyl-l-pyrroline-*N*-oxide (DMPO; Sigma, St. Louis, MO) as a spin trapping agent. DMPO was purified prior to use by adding activated carbon into DMPO solution, followed by centrifugation at 13 300 *g* for 20 min. The procedure was repeated until a clear solution was obtained [14]. The reaction mixture was added in sequence of 50 µl of serum sample, 20 µl of deionized water, 10 µl of 1.12 M DMPO, 10 µl of 25 mM ascorbic acid (Merck, Darmstadt, Germany) and 10 µl of 10 mM *t*-BuOOH (Sigma, St. Louis, MO). The mixture was transferred to a 75 µl capillary tube and inserted into an EPR sample tube (type 5D 100 mm/170 mm, Jeol Datum, Japan). EPR spectra were recorded at ambient temperature 2 min after adding of *t*-BuOOH with an X-band EPR spectrometer (E 500, Bruker, USA) equipped with ELEXSYS Super High Sensitivity Probehead cavity. EPR measurement conditions were as follows: 350.5 ± 5.0 mT central field, 100 KHz modulation frequency, 0.1 mT modulation amplitude, 10.15 mW microwave power, 60 dB gain, 41.49 s scan time and 1.28 ms time constant.

Determination of total serum iron, transferrin saturation and ferritin

Total serum iron and transferrin saturation were measured as recommended by the ICSH [15,16] using BPT (Sigma, St. Louis, MO) as a chromogen. Absorbance was measured at 535 nm with a UV-visible spectrophotometer. Serum ferritin was determined using a Ferritin kit (Diametra, Foligno, Italy).

Statistical analysis

Statistical analyses were carried out using SPSS version 11.5 (SPSS Inc. Chicago, IL). Data were analysed by Mann-Whitney U-test. Correlation between two parameters was assessed by Spearman's correlation and p-value < 0.05 is considered significant. Data are presented as mean ± SD, unless indicated otherwise.

Results

Characteristics of patients

Characteristics and iron status of mild–moderate and severe β-thal/Hb E patients are shown in Table I. The average age of the patients is not significantly different between the groups. A significantly lower haemoglobin and higher bilirubin level were observed in severe patients. Although total serum iron and serum ferritin are different between the two groups, transferrin saturation and NTBI tended to be higher in severe patients.

Serum levels of deferiprone and deferiprone-chelated iron

Time course of serum deferiprone concentration in the patients is shown in Figure 1A. Deferiprone was rapidly absorbed into the circulation and its serum level was detected within 15 min in all patients. There is no statistical difference in the maximum concentration (C_{max}) (86.0 ± 31.3 and 83.9 ± 25.6 µM) and time of maximum concentration (T_{max}) (57.3 ± 34.1 and 50.7 ± 29.0 min) between mild–moderate and severe patients.

Table I. Characteristics and serum iron status of β-thal/Hb E patients.

Parameter	Patient		*p*-value
	Mild–moderate	Severe	
n (male/female)	11 (6/5)	10 (5/5)	
Age (years)	31.8 ± 7.5	27.7 ± 8.6	0.173
Weight (kg)	50.6 ± 6.1	42.6 ± 7.6*	0.020
Haemoglobin (g/l)	73.0 ± 12.0	62.0 ± 8.0*	0.006
Total bilirubin (µM)	70.7 ± 25.6	81.9 ± 42.0	0.605
Total serum iron (µM)	36.5 ± 11.4	34.7 ± 6.9	0.918
Transferrin saturation (%)	67.4 ± 17.1	73.3 ± 12.1	0.426
Serum ferritin (ng/ml)	2763.2 (624.2–3729.6)	2754.0 (2150.6–3547.3)	0.152
Serum NTBI (µM)	1.2 ± 0.7	1.6 ± 1.3	0.605

Data are mean ± SD, except for serum ferritin, which is geometric mean (minimum–maximum).

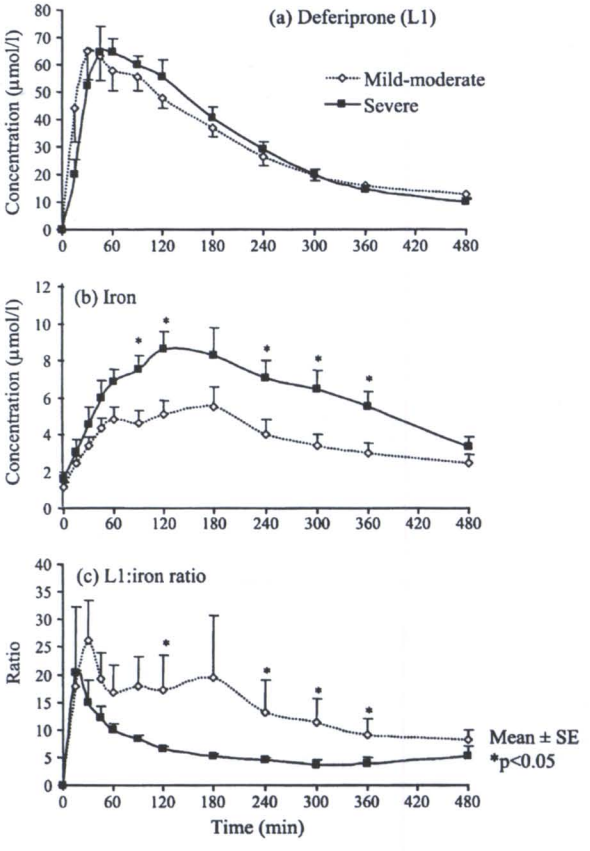

Figure 1. Time-serum concentration profile of deferiprone (A), deferiprone-chelated iron (B) and molar ratio of deferiprone to iron (C) in β-thal/Hb E patients after administration of a single oral dose of 25 mg/kg body weight of deferiprone. Measurements of deferiprone and deferiprone-chelated iron are described in Materials and methods.

Concomitant with the increase in drug serum level, there was an increase in deferiprone-chelated iron, which reached maximum concentration within 120–180 min in both groups (Figure 1B). Interestingly, in severe patients levels of deferiprone-chelated iron were significantly higher ($p < 0.05$) than in the mild–moderate group. C_{max} of deferiprone-chelated iron was 6.9 ± 3.1 μM and 9.8 ± 4.2 μM in mild–moderate and severe patients, respectively. Corresponding with increased deferiprone-chelated serum iron, the total 24-h urinary iron excretion (UIE) was also higher in severe patients (0.21 ± 0.22 and 0.36 ± 0.22 mmol for mild–moderate and severe patients, respectively). There are good correlations between C_{max} of deferiprone-chelated iron and UIE ($r = 0.647$, $p = 0.002$) and between NTBI and UIE ($r = 0.635$, $p = 0.002$).

Molar ratio of deferiprone and deferiprone-chelated iron (L1:iron)

Molar ratios of deferiprone and deferiprone-chelated iron (L1:iron) in serum samples at each time point were estimated (Figure 1C). L1:iron ratios were 5–61 and 6–16 at T_{max} of deferiprone and then declined to 1.5–18 and 1.5–10 at 480 min after dosing for mild–moderate and severe patients, respectively. A molar ratio of less than 3 was observed at 300 min after dosing in one case of mild–moderate and in five cases of severe patients. The numbers of mild–moderate and severe patients who have serum L1:iron ratios of less than 3 were three and seven cases, respectively, at 360 min after dosing and four and five cases, respectively, at 480 min after dosing.

ESR study of free radical generating activity in serum

ESR spectra of normal and thalassemic serum in the presence of *t*-BuOOH and ascorbic acid are shown in Figure 2A and B , respectively. A doublet signal with $a_H = 0.18$ mT is identified as ascorbyl radical, an intermediate oxidized product of ascorbic acid [17]. In addition, a sextet signal with $a_N = 2.37$ mT and $a_H = 1.65$ mT is a typical EPR signal for carbon-centred radical adduct of DMPO (DMPO-C adduct) [18]. This EPR signal was detected only in thalassemic serum and the signal intensity showed a good correlation with NTBI ($r = 0.542$, $p = 0.016$, Figure 3), supporting the notion that NTBI is a catalytic iron in thalassemic serum.

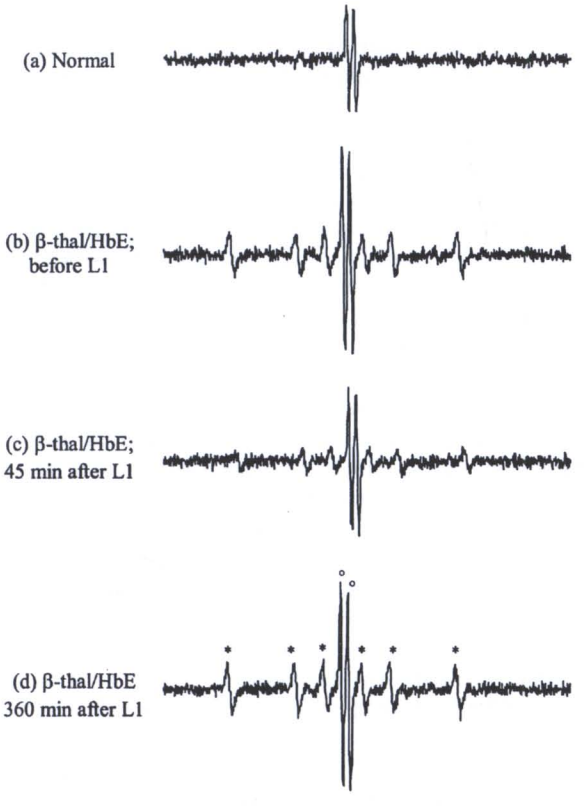

Figure 2. EPR spectrum of ascorbyl radical and DMPO-carbon centred adduct in serum of normal subject (A) and β-thal/Hb E patient before (B), 45 min (C) and 360 min (D) after administration of a single oral dose of 25 mg/kg body weight of deferiprone. (o) ascorbyl radical; (*) DMPO-carbon centred adduct.

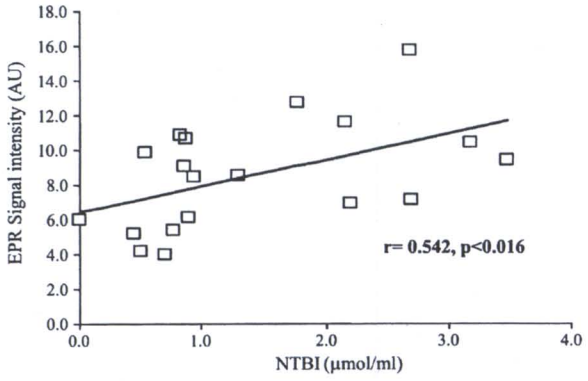

Figure 3. Correlation between non-transferrin bound iron (NTBI) and EPR signal intensity of DMPO-carbon centred adduct in serum of β-thal/Hb E patients before deferiprone administration.

Free radical generating activity of deferiprone in serum

After deferiprone administration, EPR signal intensity of DMPO-C adduct, as well as that of ascorbyl radical, were changed corresponding with the serum level of deferiprone (Figure 2C, and D and 4). These results clearly demonstrated that deferiprone inhibited free radical reaction induced in serum. At T_{max} of deferiprone, ~55% of EPR signal intensity of DMPO-C adduct was decreased. This antioxidant effect could be observed until 240–360 min after dosing. However, pro-oxidant activity as indicated by enhanced EPR signal intensity was observed in sera from a number of patients at 300, 360 and 480 min after dosing (Figure 2D). Most of these patients were in the severe group and had serum L1:iron ratio of less than 3 (Figure 5A). On the other hand, in almost all cases of mild–moderate patients a higher serum L1:iron ratio could maintain antioxidant activity over the same period of time (Figure 5B). Furthermore, iron status of the patients could also be a determining factor for deferiprone-induced free radical formation. Significant correlations are seen between NTBI and L1:iron ratio ($r = -0.455$, $p = 0.038$, at 360 min), ascorbyl radical ($r = 0.611$, $p = 0.04$ and $r = 0.517$, $p = 0.028$ at 360 and 480 min, respectively) and DMPO-C adduct ($r = 0.505$, $p = 0.023$ and $r = 0.521$, $p = 0.027$ at 360 and 480 min, respectively).

Discussion

Free radical formation induced by incomplete iron-deferiprone complexes has been under suspicion for causing serious adverse effects, particularly arthropathy and agranulocytosis. Pro-oxidant activity has been shown when deferiprone is present in low concentrations relative to iron [19,20], while higher molar ratios are redox-inactive [10]. Antioxidant activity of deferiprone has been demonstrated in several models, including iron-loaded hepatocyte

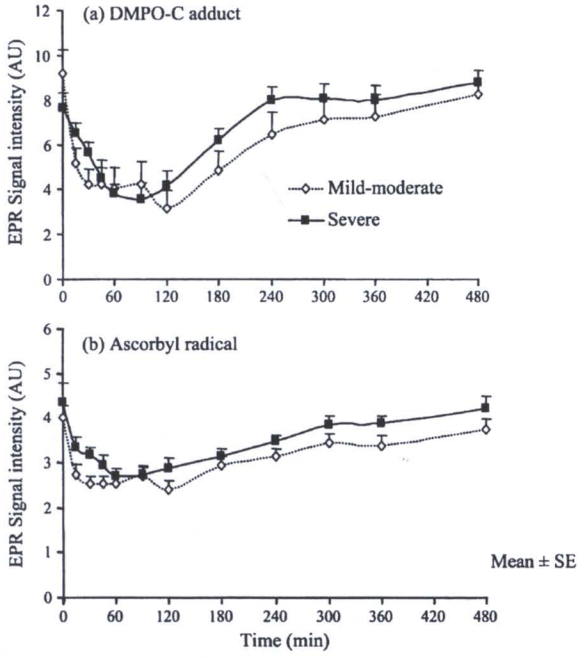

Figure 4. Time and EPR signal intensity profile of DMPO-carbon centred adduct (A) and ascorbyl radical (B) in serum of β-thal/Hb E patients after administration of a single oral dose of 25 mg/kg body weight of deferiprone.

[21], hemin-induced LDL oxidation [22] and post-ischemic cardiac injury in perfused heart [23].

In order to achieve the highest benefit of chelation therapy, the potential of free radical formation in the patients after administration of deferiprone was monitored by employing an EPR/spin trapping technique. In the presence of free radicals and catalytic transition metal, ascorbic acid undergoes one electron oxidation to give a stable ascorbyl radical ($Asc^{\cdot -}$), which can be detected by EPR at room temperature ($g = 2.00518$, $a_H = 0.176$ mT) [6,24–26]. The intensity of the $Asc^{\cdot -}$ EPR signal has been used as an indicator of oxidative stress *in vivo* and *in vitro* [24,27,28]. Without addition of *tert*-butylhydroperoxide, we observed the generation of ascorbyl radical immediately after addition of ascorbic acid into serum of thalassemic and normal subjects and also in normal serum loaded with iron. Its EPR signal intensity was also related to serum iron and iron chelator concentrations and addition of desferioxamine to sera reduced EPR signal intensity (data not shown).

EPR signal intensity of ascorbyl radical was increased in the presence of *tert*-butylhydroperoxide. Together with a spin trap, an additional EPR signal of DMPO-C adduct was detected in thalassemic, but not normal serum, indicating that thalassemic serum contains an amount of catalytic iron adequate for inducing a Fenton reaction. In addition, low levels of antioxidants in thalassemic serum could also be a factor for the presence of DMPO-C adduct. Negligible levels of serum α-tocopherol and other antioxidants

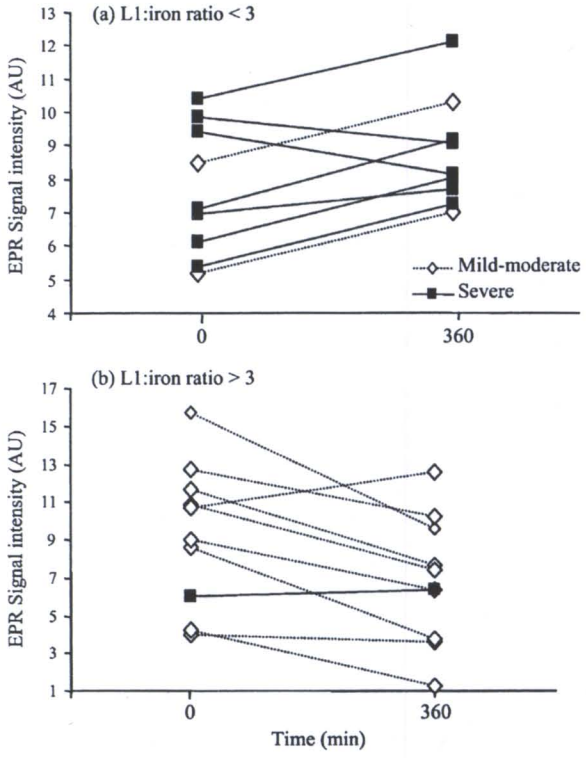

Figure 5. EPR signal intensity of DMPO-carbon centred adduct in serum of β-thal/Hb E patients before and at 360 min after administration of a single oral dose of 25 mg/kg body weight of deferiprone. Panels (A) and (B) show data from a group of patients with L1:iron ratio <3 and >3 at 360 min, respectively.

have been previously reported in thalassemia subjects [29,30].

Non-transferrin bound iron (NTBI) is believed to be the major redox active and chelatable iron in serum. NTBI has been detected in diseases associated with dysfunction of iron metabolism and also in thalassemia [30–33]. The existence of NTBI has been assumed to be a potential risk for heart and other organ damage. We have previously observed a significant correlation between NTBI and lipid peroxidation of low density lipoprotein from β-thal/Hb E patients [30]. In this study, correlation between NTBI and DMPO-C or ascorbyl radical indicated that NTBI partially contributes to the generation of free radical in serum of the thalassemic patients. Although the level of deferiprone was high, it could not completely inhibit free radical formation in thalassemic serum, indicating that there are other forms of catalytic iron in the patients. Our previous report [34] suggested that serum hemin, which is found in high amounts in thalassemic patients, contributes to free radical formation.

Time-dependent intensity of free radical formation revealed dual anti- and pro-oxidant properties of deferiprone. At a serum molar ratio of deferiprone to chelated iron greater than 3, reduced free radical productions were detected. Enhanced free radical production was obtained in most of the severe patients when serum level of deferiprone declined, particularly after 300 min of dosing. The result suggested that, in these circumstances, there was a possibility of the formation of incomplete iron-deferiprone complexes circulating in sera of patients. This phenomenon may, at least partly, help explain the incidence of deferiprone-induced agranulocytosis [35]. Moreover, in some patients, deferiprone treatment had to be terminated because of arthritis, with articular tissue damage and iron disposition observed. Our observations may also support the free radical hypothesis of deferiprone-induced arthropathy [36].

In summary, our study demonstrated that pro- and antioxidant properties of deferiprone depend on its serum concentration and, more importantly, on iron loading status of the patients. NTBI could be used as a primary predictor for both chelator efficacy and toxicity. Dosage and regiment for deferiprone treatment should be sufficient to maintain serum deferiprone to iron ratio higher than 3 in order to achieve higher efficacy and lower toxicity. A dose of deferiprone 25 mg/kg body weight used in our study is enough to induce urinary iron excretion. In addition, supplementation of vitamin E may reduce the risk of oxidative damage induced by the presence of incomplete iron-deferiprone complexes, especially in patients with severe forms of thalassemia.

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Original article **P19**

ESR Spin Labeling Studies on the Site of Iron-induced Free Radical Reaction in Low Density Lipoprotein

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Abstract

A possible mechanism to promote low density lipoprotein (LDL) oxidation, which believed to be crucial for the early event in atherogenesis, is free radical reaction. Kinetic and site of free radical reaction in the specific region, however, have not been exactly elucidated. In order to localize the specific site of LDL oxidation, paramagnetic fatty acids, 5- and 16-doxyl stearic acid (5- and 16-DS) were used to label phospholipid layer near hydrophilic surface and the deeper hydrophobic region of LDL, respectively. To induce free radical reaction in LDL isolated from healthy, normolipidemia male volunteers, either hemin or ferric nitrilotriacetate (Fe-NTA) was co-addition with either *tert*-butyl hydroperoxide (*t*-BuOOH) or hydrogen peroxide (H_2O_2). Decreasing of middle field ESR signal height was monitored versus time to calculate rate of ESR signal decay. Hemin, but not Fe-NTA, induced ESR signal decay. In the presence of 100 μM hemin coaddition with 1 mM H_2O_2, rate of ESR signal decay of 5- and 16-DS was $2.81\pm0.67 \times10^3$ and $8.08\pm2.85 \times10^3$ AU/min, respectively. Furthermore, the coaddition with 1 mM *t*-BuOOH, rate of ESR signal decay of 5- and 16-DS was $1.94\pm0.30 \times10^4$ and $3.54\pm1.72 \times10^4$ AU/min, respectively. Our results suggested that the rate of ESR signal decay could represent the rate of free radical reaction in the specific site of LDL which depended on species and concentration of iron and hydroperoxide. The deeper hydrophobic region of LDL is a primary site of LDL oxidation.

Keywords: LDL, Doxyl stearic acid, ESR spin labeling

Introduction

Free radicals are highly reactive species and may promote oxidation of proteins, peroxidation of membrane lipids, and modification of nucleic acids. An increase in the steady state levels of ROS beyond the antioxidant capacity of the organism, called oxidative stress, is encountered in many pathological conditions. Low density lipoprotein (LDL), a transport vehicle for water-insoluble lipid in the blood, is a highly compartmentalized system consisting of phospholipids, unesterified cholesterol (UC), and apolipoprotein (apoB-100) in the outer layer with cholesteryl esters (CE), triglyceride (TG), and antioxidants distributed in the core (1). The oxidation hypothesis proposes that LDL must be oxidative modification to become oxidized LDL (Ox-LDL) which believed to be crucial for the early event in the genesis of atherosclerotic lesion. Wide variety of mechanisms have been proposed for mediated LDL oxidation, including free and protein-bound metal ions (2). Recent studies investigated the iron-induced oxidative effects in LDL. These results suggested that iron-induced LDL oxidation at the hydrophobic region showed good correlation with oxidative

stress makers, such as the formation of thiobarbituric acid-reactive substances (TBARS) and conjugated diene (CD), ratio of cholesteryl linoleate (CL) to cholesteryl oleate (CO), (CL/CO), and lipid fluidity (3, 4, 5). A possible mechanism to promote LDL oxidation is free radical reaction, however, there was no directed evident showed that free radical reaction is initiated in core region of LDL. This present study designed to develop the technique of ESR spin labeling to localize the site of iron-induced free radical reaction in LDL.

Materials and methods

LDL was prepared from blood sample obtained from healthy, normolipidemia male volunteers. Blood samples were centrifuged at 2,330g at 4°C for 10 min to obtained serum. Pooled serum of volunteers were separated LDL by sequential density gradient ultracentrifugation, which is modified from Havel method. Paramagnetic fatty acids 5- and 16- doxyl stearic acid (5- and 16-DS), which dissolved in hexane to desired concentration and dry under nitrogen in clean glass tube, were used to label phospholipid near hydrophilic polar head group and the deeper hydrophobic region of LDL, respectively. Either hemin or ferric nitrilotriacetate (Fe-NTA) in various concentrations about 20 to 100 μM was added with either 1 mM *tert*-butyl hydroperoxide (*t*-BuOOH) or hydrogen peroxide (H_2O_2) to induce free radical reaction. Lipid mobility of LDL was determined by X-band ESR spectrometer with microwave frequency 9.8 GHz, power 10.13 mw, amplitude 100 kHz, and field modulation 0.25 and 0.125 for 5- and 16-DS, respectively. Decreasing of middle field ESR signal height was monitored versus time and the rate of ESR signal decay was calculated.

Results

Figure 1 shows ESR spectrum of 5- and 16-DS incorporated in LDL. Time course effect after incubation of hemin and Fe-NTA is showed in Figure 2. The addition of hydroperoxide and hemin, a lipophilic Fe^{3+}-containing protoporphyrin IX resulted in decreasing of ESR signal of both 5- and 16-DS. In contrast a water-soluble iron complex, Fe^{3+}-NTA, did not cause ESR signal decay.

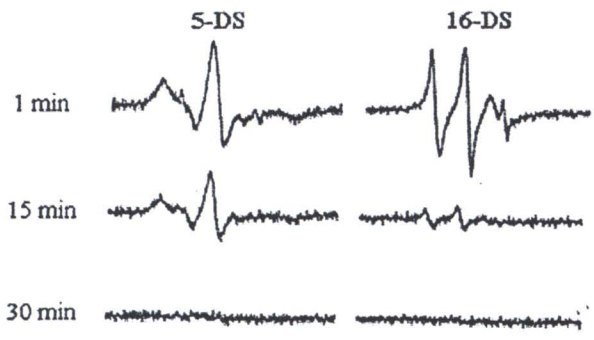

Figure 1. ESR spectrum of 5- and 16-DS in the present of hemin and peroxide.

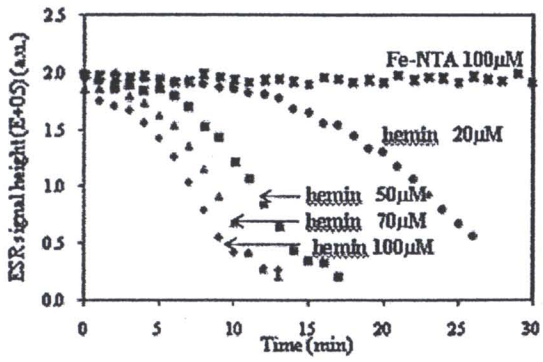

Figure 2. Time course of ESR signal decay in LDL induced by hemin and Fe-NTA coaddition with hydroperoxide.

Table 1. showed rate of ESR signal decay of 5- and 16-DS induced by hemin co-incubated with *t*- BuOOH or H_2O_2. With the same experimental condition, rate of ESR signal decay of 16-DS was faster than 5-DS. Moreover, *t*- BuOOH showed a higher potency than H_2O_2 to induce the signal decay of the both probe.

Table 1. Rate of ESR signal decay ($\times 10^4$ Au/min) of free radical reaction induced by hemin and hydroperoxide.

Hemin (μM)	5-DS		16-DS	
	t-BuOOH	H_2O_2	*t*-BuOOH	H_2O_2
20	0.84±0.30	0.07±0.06	1.49±0.57	0.49±0.29
50	1.66±0.40	0.24±0.05	2.63±1.20	0.70±0.41
70	1.77±0.44	0.27±0.08	4.14±2.09	0.80±0.36
100	1.94±0.30	0.29±0.07	3.54±1.72	0.81±0.29

Data are presented as mean ± SD of 3-independent experiments

Conclusion

Our results suggested the rate of ESR signal decay could represent the rate of free radical reaction in the specific site of LDL which depended on species and concentration of iron and hydroperoxide. It might suggest that oxidative modification of LDL via free radical reaction might initiate in the deeper hydrophobic domain.

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