A naphthoquinone derivative can induce anemia through phosphatidylserine exposure mediated erythrophagocytosis

Ji-Yoon Noh, Jong-Sook Park, Kyung-Min Lim, Keunyoung Kim, Ok-Nam Bae,
Seung-Min Chung, Sue Shin, and Jin-Ho Chung

College of Pharmacy, Seoul National University, Seoul 151-742, Korea (J.-Y.N., J.-S.P., K.-M.L., K.K., O.-N.B., S.-M.C., J.-H.C.); Department of Laboratory Medicine, Boramae Hospital, 156-707, Korea (S.S.)
Running title: Quinone-induced anemia through PS exposure

Corresponding author: Jin-Ho Chung, College of Pharmacy, Seoul National University, Seoul 151-742, Korea. Tel.: +82-2-880-7856; fax: +82-2-885-4157; E-mail address: jhc302@snu.ac.kr.

List of non-standard abbreviations

CFDA, 5-(and-6)-carboxyfluorescein diacetate; C₆-NBD-PS, 1-Palmitoyl-2-[6-[(7-nitro-2-1,3-benzoazolazol-4-yl)amino]caproyl]-sn-glycero-3-phosphoserine; C₆-NBD-PC, 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoazolazol-4-yl)amino]hexanoyl]-sn-glycero-3-phosphocholine; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MV, microvesicles; PS, phosphatidylserine

Section assignment: Toxicology
Abstract

A naphthoquinone derivative, β-lapachone (βL; 3,4-dihydro-2,2-dimethyl-2H-naphtol[1,2-b]pyran-5,6-dione), is receiving a huge attention for its potent therapeutic effects against various diseases. However, during the preclinical safety evaluation repeated oral treatment of βL in rats induced anemia, that is, a significantly decreased erythrocyte count. In this study, in an effort to elucidate the mechanism underlying the βL-induced anemia, we investigated the effects of βL on erythrocytes with freshly isolated human erythrocytes in vitro and rat in vivo. βL did not induce erythrocyte hemolysis, indicating that direct hemotoxicity was not involved in βL-associated anemia. Meanwhile, phosphatidylserine (PS) exposure along with spherocytic shape change and microvesicle (MV) generation, important factors in the facilitation of erythrophagocytosis were increased significantly by βL. The PS exposure on erythrocytes was from βL-induced reactive oxygen species (ROS) generation and subsequent depletion of reduced glutathione and protein thiol, which culminated in the modified activities of phospholipid translocases, that is, inhibition of flippase and activation of scramblase. Importantly, co-incubation of macrophage with βL-treated erythrocyte in vitro showed increased erythrophagocytosis, demonstrating that the removal of erythrocyte by macrophage can be facilitated by βL-induced PS exposure. In a good accordance with these in vitro results, after oral administration of βL in rat, increased PS exposure and depletion of GSH were observed along with enhanced splenic sequestration of erythrocytes. In conclusion, these results suggest that βL-induced anemia might be mediated through the PS exposure and subsequent erythrophagocytosis, providing a novel insight into the drug-induced anemia.
Introduction

A naturally occurring naphthoquinone, \( \beta \)-lapachone (\( \beta \)-L; 3,4-dihydro-2,2-dimethyl-2H-naphtol[1,2-b]pyran-5,6-dione) from lapacho tree (\textit{Tabebuia avellanedae}) is known to have various pharmacological activities including antiviral, antiprotozoal and anticancer effects (Dong et al., 2009; Menna-Barreto et al., 2009; Trachootham et al., 2009). These biological activities of \( \beta \)-L were suggested to be from the inhibition of DNA topoisomerase activity or the impairment of DNA repair through the reactive oxygen species (ROS) generation via NAD(P)H:quinone oxidoreductase 1 (NQO1) (Reinicke et al., 2005; Bey et al., 2007). Reduction of \( \beta \)-L by NQO1 generates unstable hydroquinone and ROS, inducing cytotoxicity against tumor cells through sulfhydryl-mediated reaction and nonspecific enzyme inhibition (Oliveira-Brett et al., 2002). Incidentally, it is well known that ROS generation and sulfhydryl modification can induce cytotoxicity and tissue injuries but few studies have been focused on the potential toxicity of \( \beta \)-L.

Recently, anti-obesity and anti-diabetic effects of \( \beta \)-L have been newly discovered (Hwang et al., 2009; Kim et al., 2009) and preclinical development of \( \beta \)-L as a new drug candidate has been initiated. However, during a routine repeat dose toxicity study, a significant reduction in erythrocyte count, anemia, was observed in the rats treated with \( \beta \)-L, raising a concern over its toxicity against erythrocytes. Conspicuously, there was no indication of bone marrow toxicity or other nonspecific toxicity in the general histopathology or blood biochemistry examination, signifying that a more complicated and specific mechanism might be involved in the \( \beta \)-L-induced anemia.

Due to the frequent exposure to oxygen-rich environment, erythrocytes often become an easy target for ROS (Banerjee and Kuypers, 2004) and ROS generating drugs (Beutler, 1969), especially in the individuals with decreased glutathione (GSH) levels resulting from the
genetic glucose-6-phosphate dehydrogenase (G6PD) deficiency (Fanello et al., 2008), chemotherapy or other pathophysiological conditions. Depletion of cellular GSH and protein thiol makes erythrocytes more susceptible to ROS, causing various abnormal conditions including hemolysis (Beutler, 1969), shape change (Lucantoni et al., 2006) and phosphatidylserine (PS) exposure (Banerjee and Kuypers, 2004). Damaged erythrocytes are engulfed and destroyed by tissue macrophages of spleen and liver, a process called erythrophagocytosis, inducing severe anemia as observed in sickle cell anemia or other disease states (Kuypers et al., 1998; de Jong et al., 2001; Lang et al., 2007).

PS exposure, an apoptotic marker on the cell surface is considered as a key signal to tissue macrophages for erythrophagocytosis (Kobayashi et al., 2007; Mercer and Helenius, 2008). The PS exposure on erythrocytes can be induced by intracellular calcium increase and the depletion of ATP and thiol-regulated modification of phospholipid translocase activity (Banerjee and Kuypers, 2004; de Jong and Kuypers, 2006; Barber et al., 2009). PS-exposing erythrocytes frequently accompany apoptotic features like spherocytic shape change and PS-bearing microvesicle (MV) generation. More importantly, through the various PS receptors or PS binding glycoprotein on tissue macrophages, PS-exposing erythrocytes could be recognized and subsequently removed by tissue macrophages through erythrophagocytosis (Zhou, 2007; Dasgupta et al., 2008).

In the current study, we discovered that βL could induce PS exposure and MV generation without hemolysis, in human and rat erythrocytes. Importantly, βL-treated erythrocytes were easily removed by macrophages through erythrophagocytosis. Its underlying mechanism was elucidated and the relevance of its finding to in vivo was examined in a repeat dose study with rats in an effort to provide a novel insight into the role of PS-exposure in drug-induced anemia.
Materials and Methods

Materials

3,4-Dihydro-2,2-dimethyl-2H-naphtol[1,2-b]pyran-5,6-dione (βL) was chemically synthesized by Mazence (Suwon, Korea). 1-Palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]-sn-glycero-3-phosphoserine (C₆-NBD-PS), and 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-glycero-3-phosphocholine (C₆-NBD-PC) were purchased from Avanti Polar Lipids (Alabaster, AL). Glutaraldehyde solution, osmium tetroxide, purified human thrombin, calcium ionophore A23187, bovine serum albumin (BSA), N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] (HEPES), sodium citrate, sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO), trichloroacetic acid (TCA), Tris-base, Tris-acetate, ATP bioluminescent assay kit, mercury orange, luminol, 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB), L-ascorbic acid, trolox, and nuclear fast red solution were obtained from Sigma Chemical Co. (St. Louis, MO). Phycoerythrin-labeled monoclonal antibody against human glycophorin A (anti-glycophorin A-RPE) was from Dako (Glostrup, Denmark) and fluo-4 acetoxyethyl ester (Fluo-4 AM) was from Molecular Probes (Eugene, OR). Fluorescein-isothiocyanate (FITC)-labeled annexin V (annexin V-FITC) was from Pharmingen (San Diego, CA) and potassium ferrocyanide was from Samchun (Seoul, Korea). 2’,7’-Dichlorofluorescein diacetate (DCFH-DA), and 5-(and-6)-carboxyfluorescein diacetate (CFDA) were obtained from Invitrogen (Eugene, OR). Dulbecco’s modified eagle medium (DMEM), DPBS, penicillin/streptomycin, and fetal bovine serum were purchased from Gibco BRL Life Technologies Inc. (Grand Island, NY).

Preparation of erythrocytes

With an approval from the Ethics Committee of Health Service Center at Seoul National
University, human blood was obtained from healthy male donors (18-25 years old) using a vacutainer with acid citrate dextrose (ACD) and a 21-gauge needle (Becton Dickinson, Franklin Lakes, NJ) on the day of each experiment. Preparation of erythrocytes was conducted according to the method previously described by Chung et al. (2007) with minor modification. Briefly, packed erythrocytes were washed 3 times with PBS and once with Ringer solution (125 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 32 mM HEPES, 5 mM glucose, pH 7.4). Washed erythrocytes were resuspended in Ringer solution to a cell concentration of 5 x 10⁷ cells/mL and final CaCl₂ concentration was adjusted to 1 mM prior to use.

Flow cytometric analysis of phosphatidylserine exposure and microvesicle generation

After erythrocytes were treated with DMSO (vehicle) or βL for 24 hr at 37°C, aliquots of blood samples were diluted 10 fold with Annexin binding buffer (125 mM NaCl, 10 mM HEPES, pH 7.4) and further incubated with following substances for 30 min at room temperature in dark. Annexin V-FITC was used as a marker for phosphatidylserine (PS), while anti-glycophorin A-RPE was used as an identifier of erythrocytes and erythrocyte derived microvesicles (MV). Negative controls for annexin V binding were stained with annexin V-FITC in the presence of EDTA instead of CaCl₂ adjusted to final 2.5 mM. The MV from erythrocytes had diameters less than 1 μm compared with the size of 1 μm-diameter standard beads in the histogram. Samples were analyzed on the flow cytometer FACScalibur™ (Becton Dickinson, San Jose, CA) equipped with argon-ion laser emitting at 488 nm. The light scatter and fluorescence channels were set on log scale. Data from 10,000 events were collected and analyzed using CellQuest™ Pro software. The extent of hemolysis was determined spectrophotometrically at 540 nm.
Microscopic observation using scanning electron microscopy and measurement of reactive oxygen species (ROS)

Erythrocytes were observed on scanning electron microscope (JEOL, Japan) according to the methods previously described by Chung et al. (2007). ROS generation was determined by chemiluminescence and dichlorofluorescein (DCF) fluorescence. For chemiluminescence, luminol (250 μM) was added to erythrocytes, and the reaction was initiated by adding βL. Chemiluminescence was monitored continuously up to 240 min using a luminometer (Auto Lumat LB953, Berthold, Germany). The total amount of chemiluminescence was calculated by integrating the area under the curve. ROS-induced DCF (dichlorofluorescein) fluorescence was measured using fluorescent probe, 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) according to the previously described method (Amer et al., 2004). Briefly, erythrocytes were preincubated at 37°C with 0.4 mM DCFH-DA for 15 min with horizontal agitation. After unloaded DCFH-DA was removed by washing with 1 mM CaCl₂ containing Ringer solution, βL was treated for a further 240 min. The aliquots were diluted appropriately and the fluorescence of intracellular DCF was measured in a FACScalibur™ (Becton Dickinson, San Jose, CA).

Measurement of glutathione (GSH) and protein thiol levels

GSH level in erythrocytes was measured according to the method previously described by Amer et al. (2004). Briefly, vehicle or βL treated erythrocytes were washed and incubated at 37°C for 3 min, with 40 μM of mercury orange. Cells were diluted in Ringer solution and analyzed by FACScalibur™ (Becton Dickinson, San Jose, CA).

Protein thiol concentrations were measured by a modified assay based on the previously
described colorimetric method (Di Monte et al., 1984). One mL of βL-treated erythrocytes was centrifuged at 7,000 g for 1 min, and pellet was resuspended with lysis buffer (5 mM sodium phosphate, pH 8) and incubated on ice for 30 min. Total lysate was resuspended with 5% perchloric acid on the ratio of 2 to 5, and then centrifuged at 7,000 g for 2 min. The pellet was solubilized in 1 mL of Tris-EDTA buffer (0.5 mM Tris-HCl, 5 mM EDTA, pH 7.6) containing 1% SDS. DTNB (250 μM) was added to the samples, and the change of the absorbance was measured at 412 nm. The content of protein thiol was calculated on the basis of a glutathione calibration curve and divided by the protein content, which was measured by Bio-Rad protein assay kit (Hercules, CA).

**Measurement of phospholipid translocation**

Phospholipid translocation was measured according to the methods previously described (Hilarius et al., 2004). Briefly, erythrocytes (5×10^7 cells/mL) were incubated with βL and then loaded with 0.5 μM C6-NBD-PS or C6-NBD-PC. Aliquots from the cell suspension were removed at the indicated time intervals and placed on ice-cold Ringer solution for 10 min in the presence or absence of 1% bovine serum albumin (BSA) respectively. The amount of internalized probe was determined by comparing the fluorescence intensity associated with the cells before and after back-extraction. Samples were analyzed on the flow cytometer FACScalibur™ (Becton Dickinson, San Jose, CA).

**Measurement of intracellular ATP and calcium level**

The measurement of ATP and calcium levels was simply modified from the methods previously described by Chung et al. (2007). Samples for detection of intracellular ATP level were adapted to luciferin/luciferase assay in Luminoskan (Labsystems, Franklin, MA)
using an ATP assay kit (Sigma Chemical Co., St. Louis, MO). For detecting intracellular calcium increase, erythrocytes were loaded with 3 μM Fluo-4 AM for 1 hr at 37°C in the dark. After βL were incubated for indicated time, samples were analyzed on the flow cytometer FACScalibur™ (Becton Dickinson, San Jose, CA).

Measurement of in vitro erythrophagocytosis

Mouse macrophage cell line, RAW 264.7 were purchased from The American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37°C under 5% CO₂ atmosphere. RAW 264.7 cells were seeded (1 × 10⁶ cells/well) to 6-well plate for 24 hr, followed by co-incubation for 18 hr with 200 μL of vehicle- or βL-treated rat erythrocytes (2 × 10⁷ cells/well) and then observed with phase-contrast microscopy (Olympus IX70, Japan). Chemical treated erythrocytes were washed and resuspended with DMEM and then co-cultured with macrophages at 37°C under 5% CO₂ atmosphere. For flow cytometric analysis, chemical treated erythrocytes were loaded with 10 μM of CFDA for 30 min before co-incubation. After the co-incubation, macrophages were harvested and washed for several times to remove unbound erythrocytes. Samples were analyzed on the flow cytometer FACScalibur™ (Becton Dickinson, San Jose, CA). Data from 50,000 events were collected and analyzed using CellQuest™ Pro software.

In vivo experiments

Detection of PS exposure following βL administration in rats. All of the protocols used in vivo experiments were approved by the Ethics Committee of Animal Service Centre at Seoul National University. Male Sprague-Dawley rats (Samtako Co., Osan, Korea)
weighing 200-250 g were used in all experiments. Before the experiments, animals were acclimated for 1 week. Food and water were provided ad libitum. At the time of experiment, rats were randomly grouped for control (saline, containing 1% sodium lauryl sulfate), 25, 75, and 200 mg/kg of βL doses. Four hours after βL was administered per oral, blood was collected from the abdominal aorta using 3.8% trisodium citrate as anticoagulant. An aliquot of blood sample was diluted 200 fold with the following buffer (10 mM HEPES-Na, 136 mM NaCl, 2.7 mM KCl, 2.0 mM MgCl₂, 1.0 mM NaH₂PO₄, 5.0 mM dextrose, 5 mg/ml BSA, 2.5 mM CaCl₂, pH 7.4) and was stained with annexin V-FITC for 15 min in the dark. PS exposure was measured as described above.

Hematoxylin and eosin staining and Prussian blue staining of organs. Liver and spleen were obtained surgically after blood collection, weighed and fixed with 10% formalin. The tissue specimens were cut into 4-μm-thick sections and stained with hematoxylin and eosin (H&E) and ferrocyanide. After counterstaining with nuclear fast red solution, the ferrocyanide stained slides were dehydrated and mounted. The slides were observed with bright-field microscope (Olympus CX41, Japan).

Statistical analysis

The means and standard errors of means were calculated for all treatment groups. The data were subjected to one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test or student t-test to determine which means were significantly different from the control. In all cases, a p value of < 0.05 was used to determine significant differences.
Results

During the preclinical development, a significant reduction in erythrocyte counts was observed in the rats treated with βL for 2 weeks (Fig. 1A). However, in freshly isolated human erythrocytes, even the high concentration of βL did not induce direct hemolysis (Fig. 1B). In contrast, phosphatidylserine (PS) exposure, an important pathological change of erythrocytes, was significantly enhanced by βL treatment in concentration- and time-dependent manners (Fig. 1C and 1D).

Along with the PS exposure, βL caused the release of erythrocyte membrane-derived microvesicles (MVs) in concentration- and time-dependent manners (Fig. 2A, 2B, and 2C). In line with the MV generation, βL-treated erythrocytes displayed echinocytes (Fig. 2D, arrow), cells with protruded surface, and MV-shed spherocytes (Fig. 2D, arrowhead), while vehicle-treated erythrocytes showed a normal discocytes. As shown in Fig. 2E, most of βL-generated MV (99%) also expressed PS on their surfaces, which can be also engulfed by macrophages (Willekens et al., 2005).

βL-induced cytotoxicity is mainly mediated by oxidative stress through ROS generation (Reinicke et al., 2005). To investigate if ROS can be induced by βL, erythrocytes were incubated with βL and ROS was measured by DCF fluorescence and luminol-enhanced chemiluminescence. As shown in Fig. 3A and 3B, βL increased ROS generation in a concentration-dependent manner. In accordance with the ROS generation, βL caused the depletion of GSH (Fig. 3C) and protein thiol (Fig. 3D) which is critical in the maintenance of cellular homeostasis when cells are exposed to the oxidative stress. To clarify the role of cellular ROS and protein thiol in βL-induced erythrocyte PS exposure, antioxidants and thiol donor were pre-treated and PS exposure was measured. Both antioxidants and thiol donor
could reverse βL-induced PS exposure (Fig. 3E), suggesting that the thiol depletion from βL-induced ROS generation play an important role in the disturbance of PS asymmetry.

The depletion of intracellular protein thiol can activate scramblase and inhibit flippase which induces and maintains PS on the outer membrane, respectively (Tyurina et al., 2007). Consistently, after βL treatment, the scramblase was activated, as measured by increased inward translocation of C₆-NBD PC (Fig. 4A), and the activity of flippase was inhibited as determined by decreased inward translocation of C₆-NBD PS (Fig. 4B). In addition to the depletion of protein thiol, flippase can also be affected by the depletion of intracellular ATP. As a result, βL was shown to induce ATP depletion in a concentration-dependent manner (Fig. 4C). Interestingly, intracellular calcium level, another important mediator of scramblase activation and flippase inhibition, was not increased in βL-treated erythrocytes (Fig. 4D).

PS exposure on erythrocytes can be a signal for the recognition and subsequent erythrophagocytosis by tissue macrophages. First, we confirmed that the βL-induced PS exposure was similar in rat erythrocytes to human erythrocytes (data not shown). To investigate the erythrophagocytosis, βL-exposed rat erythrocytes were co-incubated with murine macrophage cell line RAW 264.7 cells for 18 hr. As shown in Fig. 5A, βL-exposed erythrocytes were bound and engulfed by macrophages. The erythrophagocytosis by βL was further confirmed by significantly increased fluorescent macrophages resulting from phagocytosis of CFDA-loaded erythrocytes (Fig. 5B) which was in a good correlation with βL-induced PS exposure (Fig. 1C).

The effects of βL on PS exposure and splenic sequestration were examined in rats in vivo. Four hours after the oral administration of 0, 75 or 200 mg/kg of βL (5 mL/kg), blood samples were collected to determine PS exposure and GSH levels on erythrocytes. βL indeed induced PS exposure and GSH depletion in vivo (Fig. 6A), while erythrocyte integrity,
as assessed by osmotic fragility-induced hemolysis was not affected (data not shown), in a good accordance with in vitro data. To explore the long-term consequences of the βL-induced erythrocyte PS exposure, rats were administered with βL once daily for 2 weeks; the time when anemia was observed. The relative organ weights of both liver and spleen were increased significantly (Fig. 6B, upper), and more importantly, the spleen of βL-treated rats was accumulated with iron as determined by increased blue-staining with Prussian blue (Fig. 6B, lower), suggesting that βL accelerated erythrocyte clearance indeed through erythrophagocytosis.
Discussion

In the present study, we demonstrated that βL could induce anemia indirectly by enhancing erythrophagocytosis and subsequent splenic sequestration of erythrocytes through phosphatidylserine (PS) exposure without direct cytotoxicity on erythrocytes. βL-enhanced PS exposure was accomplished from the activation of scramblase and the inhibition of flippase by ROS generation and the depletion of GSH and protein thiol. The βL-treated erythrocytes also displayed abnormal shape and MV generation, which could further contribute to the erythrophagocytosis by tissue macrophages. These in vitro observations were confirmed in rat in vivo as determined by increased splenic sequestrations of erythrocytes in βL-treated rats. This is the first study showing that drug-induced PS exposure in erythrocytes can induce increased erythrocyte clearance and ultimately anemia, to our best knowledge.

βL and its derivatives are getting a huge interest for potent therapeutic activities in various diseases including cancer, obesity, and cardiovascular diseases (Dong et al., 2009; Hwang et al., 2009; Kim et al., 2009). Notably, ROS generating capability is suggested as a key mode of action for both therapeutic and adverse effects of βL (Reinicke et al., 2005; Bey et al., 2007). Considering the vulnerability of erythrocytes to oxidative stress, toxicity of βL to erythrocytes could be easily foreseeable. Recent finding of Miao et al. (2008) that a βL derivative could be rapidly sequestered into and metabolized in erythrocytes, further support the specific and selective toxicities of βL to erythrocyte. Besides βL, many ROS generating drugs are being developed or marketed for therapeutic uses in cancer and other diseases (Davis et al., 2001; Fibach and Rachmilewitz, 2008; Trachootham et al., 2009).
conjunction with our findings with βL, we suggest that a serious attention should be paid for the potential toxicity of the ROS generating drugs to erythrocytes.

The maintenance of glutathione (GSH) level is vital for the protection of erythrocytes from ROS. Exemplifying this, in glucose-6-phosphate dehydrogenase (G6PD) deficiencies where sufficient NADPH is not provided for the maintenance of reduced GSH, erythrocytes were vulnerable to oxidative hemolysis by ROS generating drugs, such as glibenclamide, doxorubicin, dapsone, and so on (Mason et al., 2007; Ganesan et al., 2009). Excessive ROS and resultant depletion of GSH could alter the activities of vital enzymes or proteins that need reduced cysteine residue and free thiol for their activity (Devaux, 1988; de Jong and Kuypers, 2006), bringing about erythrocyte damages. In the present study, we demonstrated that βL-induced ROS could inhibit flippase and activate scramblase, perturbing the membrane phospholipid asymmetry and inducing PS exposure.

PS exposure is a key signal for the erythrocyte clearance by tissue macrophages (Mandal et al., 2005; Tyurina et al., 2007; Fairn and Grinstein, 2008) and novel PS receptors on splenic macrophages are being identified such as Tim4 (T-cell immunoglobulin- and mucin-domain-containing molecule) (Kobayashi et al., 2007; Miyanishi et al., 2007; Park et al., 2007) and PS binding glycoprotein, lactadherin (Dasgupta et al., 2008). Role of PS exposure is well established in the anemia associated with genetic or pathologic disease states but its implications in chemical-induced anemia have not been fully understood. Recently, Lang et al. (2008) suggested the possible contribution of erythrocyte PS exposure and eryptosis, apoptotic features in erythrocyte in lead-induced anemia through in vitro experiments. However, the clear demonstration of their in vitro results in relevant in vivo model has not been provided yet. Our study showing that βL could induce anemia through
increased PS exposure and erythrophagocytosis in vivo, provides an important piece of evidence for the contribution of PS exposure in chemical-induced anemia.

Recently, Bowman et al. (2005a) reported that 5-hydroxyprimaquine (5-HPQ), a metabolite of primaquine could enhance the clearance of rat erythrocyte by splenic macrophage. They found that 5-HPQ-induced ROS could generate hemoglobin thyl radical and adduct with membrane protein and suggested that oxidative damages of membrane cytoskeleton and CD47 might increase erythrophagocytosis in rat (Bowman et al., 2005b). However, they could not find PS exposure following 5-HPQ treatment in erythrocytes. The discrepancy from our findings might be explained by the difference in species, that is, between rat and human in our study or the chemicals used. We confirmed that βL could induce PS exposure significantly in rat erythrocytes (data not shown), suggesting that the difference in chemicals is the most probable reason underlying the discrepancy from our study.

In conclusion, we demonstrated that βL-induced anemia might be mediated by PS exposure and increased erythrophagocytosis by spleen through in vitro and in vivo models. Most importantly, this study has provided an important clue for the role of PS exposure and erythrophagocytosis in the drug-induced anemia.
References

Blood 98:1577-1584.


FOOTNOTES

These authors (J.-Y.N. and J.-S.P.) contributed equally.

This work was partially supported by the SRC/ERC program of MOST/KOSEF (R11-2007-107-01002-0).
Legends for Figures

Figure 1. Phosphatidylserine (PS) exposure by βL in human erythrocytes. A, βL was administered to SD rats once daily for two weeks per oral and blood cell counts were measured. After human erythrocyte suspension was incubated with DMSO (vehicle control) or 10, 25, 50 μM of βL for 24 hr at 37°C, B, released hemoglobin due to hemolysis was measured, and C, flow cytometric analysis was done. Five μM of mercury chloride was used as a positive control for hemolysis. With representative histograms of erythrocyte suspension treated with DMSO or 50 μM of βL, percentage of cell expressing phosphatidylserine (PS) by dose dependency (C, inset) and time dependency (D) were shown. Values are mean ± SEM of 4 to 5 independent experiments from different blood donors. * significant differences from control group (p<0.05).

Figure 2. Effects of βL on microvesicle (MV) generation in human erythrocytes. After erythrocytes were treated with DMSO or 10, 25, 50 μM of βL for 24 hr at 37°C, the extent of MV generation was measured by flow cytometry. A, Erythrocytes and MVs were identified by forward scatter characteristics in a representative dot plot. B and C, Dose- and time-dependent MV release from erythrocytes were measured. Values are mean ± SEM of 4 to 5 independent experiments from different blood donors. * significant differences from control group (p<0.05). D, After treatment, the cells were fixed and the morphological changes were examined using scanning electron microscope. Representative data of three independent experiments from different blood donors was shown here (scale bar: 5 μm). E, Histogram of PS exposure on MV generated by βL was represented using FITC-labeled-
annexin V.

**Figure 3.** Effects of βL on ROS generation and depletion of glutathione (GSH) and protein thiol in human erythrocytes. After erythrocytes were incubated with various doses of βL for 4 hr, A, ROS generation was measured by flow cytometric analysis of dichlorofluorescein (DCF) fluorescence and, B, luminol-enhanced chemiluminescence. C, intracellular GSH level was detected by flow cytometric analysis of intracellular GSH bound mercury orange. D, after erythrocytes were incubated with βL for 24 hr, protein thiol content in erythrocytes was measured by a colorimetric method using DTNB. E, The effects of various inhibitors on PS exposure was determined with 100 μM trolox, 50 μM vitamin C, and 1 mM DTT for 15 min treatment prior to 50 μM of βL for 16 hr. Values are mean ± SEM of 3 to 4 independent experiments from different blood donors. * significant differences from control group, and # significant differences from βL alone group (p<0.05).

**Figure 4.** Effects of βL on phospholipid translocation and intracellular ATP and calcium levels in human erythrocytes. Erythrocytes were treated with various doses of βL for 24 hr at 37°C, and then the extents of phospholipid translocation were measured. A, Activated C₆-NBD PC translocation by scramblase was determined in βL-treated erythrocytes. B, Inhibited C₆-NBD PS translocation by flippase was measured as described in Methods. C, The levels of intracellular ATP was measured by luciferin-luciferase assay. D, After erythrocytes loaded with fluo-4 were treated with DMSO (control) or βL for 24 hr, intracellular calcium levels were evaluated by fluorescent cells using flow cytometer. Values are mean ± SEM of 3 independent experiments. * significant differences from
control group (p<0.05).

**Figure 5. Enhancement of erythrophagocytosis by βL-treated erythrocytes.** After rat erythrocytes were treated with DMSO or 25, 50 μM of βL for 24 hr at 37°C, cells were stained with 10 μM of CFDA for 30 min in-between the washing steps and then co-cultured with RAW 264.7 cells for 18 hr at 37°C. **A,** Representative microphotograph of βL-induced erythrophagocytosis (lower, arrow; original magnification: x 400), and **B,** the numbers of fluorescence positive RAW 264.7 cells induced by engulfment of CFDA-loaded erythrocytes were detected using flow cytometer. * significant differences from control group (p<0.05).

**Figure 6. Increase of PS exposure and erythrophagocytosis in rats in vivo by βL administration.** **A,** Extents of PS exposure (upper) on erythrocytes and levels of GSH (lower) were measured from whole blood using flow cytometry, 4 hr after oral administration of saline (vehicle), 75, and 200 mg/kg of βL. Values are mean ± SEM of 4 to 6 animals. **B,** After oral administration of saline, 25, 75, and 200 mg/kg/day of βL for 2 weeks, liver and spleen were isolated and weighed (upper), followed by fixation for Prussian blue staining. Representative microphotograph of spleen from control (lower, 1 and 3) or 200 mg/kg/day of βL treated group (lower, 2 and 4). The original magnifications were 100-fold for the picture of 1 and 2, and 400-fold for 3 and 4. Values are mean ± SEM (N = 6). * significant differences from control group (p<0.05).
Table 1: Effects of dose on hematocrit and RBC counts.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Male RBC (× 10⁶/μL)</th>
<th>Male HCT (%)</th>
<th>Female RBC (× 10⁶/μL)</th>
<th>Female HCT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.69 ± 0.17</td>
<td>41.8 ± 0.84</td>
<td>6.92 ± 0.14</td>
<td>41.8 ± 0.49</td>
</tr>
<tr>
<td>100</td>
<td>6.45 ± 0.07</td>
<td>39.8 ± 0.31</td>
<td>6.40 ± 0.17</td>
<td>39.2 ± 1.16</td>
</tr>
<tr>
<td>200</td>
<td>5.77 ± 0.11*</td>
<td>37.7 ± 0.84*</td>
<td>5.35 ± 0.04*</td>
<td>36.3 ± 1.02*</td>
</tr>
<tr>
<td>400</td>
<td>5.21 ± 0.18*</td>
<td>36.5 ± 1.15*</td>
<td>5.10 ± 0.16*</td>
<td>39.0 ± 1.05</td>
</tr>
</tbody>
</table>

(Mean ± S.E.M.)

Figure 1

Panel A: Table showing changes in hematocrit and RBC counts with dose.

Panel B: Graph showing percentage of hemolysis with different concentrations of βL.

Panel C: Flow cytometry data showing Annexin-V positive red cells with control and βL treatment.

Panel D: Graph showing time course of Annexin-V positive red cells with control and βL treatment.
Figure 2
Figure 3
Figure 4
Figure 5

A

Control

βL

B

Positive RAW 264.7 cells (x10^3)

βL (μM)

0 25 50

*
Figure 6