Biochemistry and Biophysics Reports 10 (2017) 26–31

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Protoporphyrin IX regulates peripheral benzodiazepine receptor associated protein 7 (PAP7) and divalent metal transporter 1 (DMT1) in K562 cells

Yasumasa Okazaki*, Jonathan Glass**

Feist-Weiller Cancer Center and the Department of Medicine, Louisiana State University Health Sciences Center-Shreveport, Shreveport, LA 71130, USA

ARTICLE INFO

Keywords:
Peripheral-type benzodiazepine receptor (PBR)-associated protein 7 (PAP7)
Divalent metal transporter 1 (DMT1)
Peripheral-type benzodiazepine receptor (PBR)
Protoporphyrin IX
Iron metabolism

ABSTRACT

Background: Protoporphyrin IX (PP IX), the immediate precursor to heme, combines with ferrous iron to make this product. The effects of exogenous PP IX on iron metabolism remain to be elucidated. Peripheral-type benzodiazepine receptor (PBR) is implicated in the transport of coproporphyrinogen into the mitochondria for conversion to PP IX. We have demonstrated that PBR-Associated Protein 7 (PAP7) bound to the Iron Responsive Element (IRE) isoform of divalent metal transporter 1 (DMT1). PP IX and PAP7 are ligands for PBR, thus, we hypothesized that PAP7 interact with PP IX via PBR.

Methods: We have examined in K562 cells, which can be induced to undergo erythroid differentiation by PP IX and hemin, the effects of PP IX on the expression of PAP7 and other proteins involved in cellular iron metabolism, transferrin receptor 1 (TfR1), DMT1, ferritin heavy chain (FTH), c-Myc and C/EBPα by western blot and quantitative real time PCR analyses.

Results: PP IX significantly decreased mRNA levels of DMT1 (IRE) and (non-IRE) from 4 h. PP IX markedly decreased protein levels of C/EBPα, PAP7 and DMT1. In contrast, hemin, which like PP IX also induces K562 cell differentiation, had no effect on PAP7 or DMT1 expression.

Conclusion: We hypothesize that PP IX binds to PBR displacing PAP7 protein, which is then degraded, decreasing the interaction of PAP7 with DMT1 (IRE) and resulting in increased turnover of DMT1.

General significance: These results suggest that exogenous PP IX disrupts iron metabolism by decreasing the protein expression levels of PAP7, DMT1 and C/EBPα.

1. Introduction

Much of the iron taken up by cells via the endocytosis of transferrin is incorporated into heme-containing proteins, particularly hemoglobin, myoglobin, and the various cytochromes. In the biosynthesis of heme [1], the synthesis of protoporphyrin IX (PP IX) from coproporphyrinogen III is catalyzed by coproporphyrinogen oxidase (CPO), an enzyme located in the mitochondrial intermembrane space in mammals. The transport of coproporphyrinogen into the mitochondria is possibly mediated by the peripheral-type benzodiazepine receptor (PBR) [2–4]. PBR has been implicated as being involved in a number of physiologic states including steroidogenesis and pathologic conditions including cancer [5]. Multiple ligands for PBR have been defined such as benzodiazepine derivatives and endogenously generated compounds including the dicarboxylic porphyrins such as PP IX [4], and various protein ligands including the 32-kDa voltage-dependent anion channel (VDAC), PBR-associated protein 1 (PRAX-1), PBR and protein kinase A (PKA) regulatory subunit RIa-associated protein (PAP7) [6,7]. Because of its fluorescence properties, PP IX is used for photodynamic diagnostic and therapeutic purposes for cancer. PP IX levels sufficient for these purposes may be achieved by the endogenous production or administration of the porphyrin or, especially for superficial tumors, by the application of the PP IX precursor 5-aminolevulinic acid [8–10]. The therapeutic effects of PP IX can be enhanced by silencing ferrochelatase, which catalyzes insertion of iron into PP IX [10]. Understanding the relation between altered levels of PP IX and cellular iron metabolism may disclose new insights that be able to enhance the therapeutic value of photodynamic therapy for cancer.

We have identified a protein that bound to the C-terminus of the Iron Responsive Element (IRE) containing isoform of Divalent metal transporter 1 (DMT1) from a rat intestinal cDNA library using the yeast two hybrid system. This protein was initially named DMT1 Associated Protein or DAP (AY336075) and is 97.5% identical to the previously described peripheral-type benzodiazepine receptor (PBR)-associated

* Corresponding author. Present address: Department of Pathology and Biological Responses, Nagoya University Graduate School of Medicine, 65 tsurumai-cho, Showa-ku, Nagoya, 466-8550, Japan.
** Corresponding author.
E-mail addresses: samasuya@med.nagoya-u.ac.jp (Y. Okazaki), jglass1063@gmail.com (J. Glass).

http://dx.doi.org/10.1016/j.bbrep.2017.02.007
Received 21 November 2016; Received in revised form 17 February 2017; Accepted 25 February 2017
Available online 27 February 2017

protein 7 (PAP7) [11]. DMT1 is the transporter of ferrous ions across the brush border membrane of the duodenal epithelium and also across endosomal membranes for iron transport into cells via the transferrin-transferrin receptor pathway following reduction by STEAP3 [12–14]. The accumulation of PP IX causes neurotoxicity and the interaction of PAP7 and DMT1 was shown in PC12 cell line [15], suggesting a pivotal role in iron metabolism for PAP7 and PP IX in neurons. In the small intestine, the pharmacological characterization of the binding affinity of PBR for PP IX differs among the duodenum, jejunum, and ileum [16]. PP IX was shown to displace the binding of PK11195 and PBR [4], suggesting PP IX disrupts the interaction between PBR and other proteins. As PAP7 binds to DMT1 and presumably binds to PBR, it was of interest to see if PP IX that bound to PBR would affect PAP7 levels and as a consequence affect other aspects of cellular iron metabolism.

To begin these studies, we examined the effects of PP IX on PAP7 and other proteins involved in cellular iron metabolism using the K562 cell line as a model system. Erythroid differentiation of K562 cells can be induced by a variety of agents including heme and PP IX [17,18]. In these studies, we examined the effects of various concentrations of PP IX added to K562 cells grown under different iron conditions to determine the effects of PP IX on PAP7 expression as well as on the expression of the iron related proteins, transferrin receptor 1 (TfR1), ferritin heavy chain (FTH), DMT1, and Hemoglobin F (γ-globin) as a marker of erythroid differentiation.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies against DMT1 (pan isoform), DMT1 (Iron Responsive Element, IRE isoform) and PAP7 were described previously [11,19]. Antibodies against Transferrin Receptor 1 (TfR1) and c-Myc were from Invitrogen (Carlsbad, CA, USA). Anti-actin antibody was from Sigma-Aldrich (St. Louis, MO, USA). Anti-ferritin heavy chain (FTH) antibody was from Abcam (Cambridge, MA, USA). Anti-hemoglobin F (γ-globin) antibody was from Calbiochem (San Diego, CA, USA). Anti-transferrin antibody was from Dako (Carpinteria, CA, USA). Horseradish peroxidase labeled anti-sheep IgG was from Pierce (Rockford, IL, USA). Anti-C/EBPα antibody, horseradish peroxidase labeled anti-rabbit and anti-mouse IgG antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Precision Plus Protein Standards was from Bio-Rad Laboratories, (Hercules, CA, USA). The BCA protein assay kits, NE-PER Nuclear and cytoplasmic fraction extraction reagents and Supersignal West Pico Substrate were from Pierce. TRIZOL, Superscript III first strand synthesis system for RT-PCR and SYBR Green qPCR SuperMix for iCycler instrument were from Invitrogen. DNA oligonucleotides were from IDT (Coralville, IA, USA). Fetal Bovine Serum (FBS) was from Atlanta Biologicals (Lawrenceville, GA, USA). RPMI 1640 was from Mediatech (Herndon, VA, USA). Ferric ammonium citrate (FAC) was from Fisher scientific (Pittsburgh, PA, USA). Holo-transferrin (Tf), bovine serum albumin (BSA), selenium dioxide (SeO_2), protease inhibitor cocktail, hemin and protoporphyrin IX (PP IX) were from Sigma-Aldrich.

2.2. Culture conditions for K562 cells

K562 cells (CCL-243) were obtained from ATCC (American Type Culture Collection, Rockville, MD, USA) and were grown in RPMI 1640 with 10% FBS and antibiotics (100 U/ml of penicillin G, 100 μg/ml of streptomycin). Prior to experiments, the medium was changed to serum free medium with BSA (4 mg/ml), holo-Tf (50 μg/ml), insulin (5 μg/ml), and SeO_2 (30 nM), then cells were incubated for overnight. PP IX, hemin and FAC were added for the stated times. For high iron concentration, FAC was added at 50 μM, and for low iron concentration, deferoxamine mesylate (DFO) was added at 50 μM for each well. PP IX was dissolved in DMSO. Final concentration of DMSO in medium was 0.5% (v/v). Hemin was dissolved in HCl, neutralized by NaOH and buffered in PBS.

2.3. Determination of PP IX and heme concentration in K562 cells

After exposure of K562 cells to PPlX, intracellular levels of PPIX and heme were determined by fluorescence spectrophotometry using a Synergy 2 fluorometer (Bio-Tek, Winooski, VT, USA) [2]. Briefly, cells were washed with PBS 4 times, the cell pellets vortexed vigorously with ethylacetate-acetic acid solution (3:1, v/v) and the porphyrins extracted into an equal volume of 0.1 N HCl. After vortexing and centrifugation at 12,000*g* for 1 min, the upper phase was removed and evaporated under vacuum. The components were suspended in 2 M oxalic acid, then, heated at 110 °C for 30 min to convert heme to PP IX, and the amount of PP IX determined using 410 nm as the excitation and scanning from 650 to 700 nm. The lower phase of the original ethylacetate-acetic acid/HCl extraction was centrifuged at 12,000*g* for 5 min to remove precipitated proteins, vortexed with 0.5 N HCl to extract PP IX which was again determined by fluorescence spectrophotometry. The heme and PPIX were quantified by the use of standard PPIX and hemin solutions.

2.4. Western blotting

Whole cell lysates were prepared from K562 cells lysed in RIPA buffer, as previously [11]. Nuclear extracts from K562 cells were prepared, using NE-PER Nuclear extraction reagents according to manufacturer's protocol. The membranes were subsequently incubated with the following primary antibodies with dilutions in parentheses and optimal conditions: c-Myc (1:1000), TfR1 (1:10,000), FTH (1:5000), HbF (γ-globin) (1:3000), DMT1 (pan isoform) (1:3000) and DMT1 (IRE isoform) (1:5000) in 5% skim milk overnight; PAP7 (1:5000) and actin (1:5000) in 5% skim milk 3 h; Tf (1:2000) in PBS-T 2 h and C/EBPα (1:500) in PBS-T overnight. The membranes were washed three times for 5 min each with PBS-T, and incubated with the appropriate horseradish peroxidase-labeled secondary antibodies in 5% skim milk for 1 h at room temperature. The immunoreactive proteins were detected by chemiluminescence.

2.5. Quantitative real time PCR (qRT-PCR)

RNA was extracted from K562 cells by TRIZOL and cDNA was synthesized from 5 μg of total RNA by Superscript III first strand system for RT-PCR, according to the manufacturer's protocol. The expression levels of mRNA were determined by quantitative real time PCR with an iCycler (Bio-Rad Laboratories, CA, USA), as previously [11].

3. Results

3.1. Protoporphyrin IX (PP IX) and heme concentrations in K562 cells after incubation with PP IX and hemin

Intracellular concentrations of PP IX and heme were measured after the addition of PP IX or hemin for 24 h to K562 cells cultured previously either in media of high, normal, or low iron conditions (Fig. 1A, S1A and B). Low levels of PP IX were found in the control cells and cells treated with vehicle (DMSO). The addition of hemin or Ferric Ammonium Citrate (FAC) did not significantly affect cellular levels of PP IX and heme. However, about 10 and 20 fold more PP IX was detected in cells exposed to 2.5 μM and 5 μM of PP IX respectively compared to the control cells. Interestingly, when cells were exposed simultaneously to 50 μM of hemin and 5 μM of PP IX, the amount of PP IX found in the K562 cells decreased to approximately the same level obtained with 2.5 μM of PP IX alone. The addition of PP IX also markedly increased the intracellular heme concentration about 20-fold

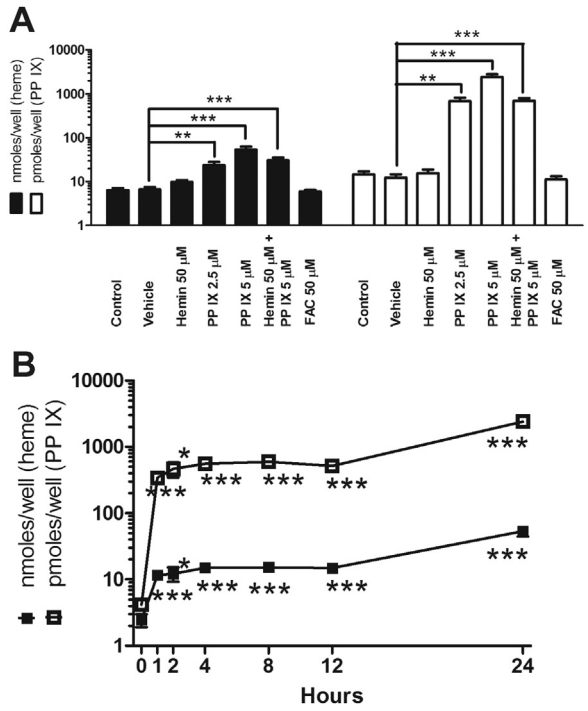

Fig. 1. Protoporphyrin IX (PP IX) and heme concentrations in K562 cells after incubation with PP IX and hemin. (A) As detailed in the Methods, K562 cells grown overnight in serum free medium in 6-well plates were treated with medium only (Control), Vehicle (DMSO), Hemin 50 μM, PP IX 2.5 μM, PP IX 5 μM, Hemin 50 μM + PP lX 5 μM, or Ferric Ammonium Citrate (FAC) 50 μM for 24 h. The concentrations of PP IX and heme in the cells were measured. **(B)** Cells were grown in serum free medium overnight, then exposed to 5 μM of PP IX and harvested at 1, 2, 4, 8, 12 and 24 h after addition of PP IX. Shown are the mean levels of PP IX as pmoles ± SEM and heme as nmoles per well ± SEM of five experiments for heme (▬) and PP IX (□). An unpaired *t*-test was applied to the results with significant differences (*; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.0001$) from vehicle alone.

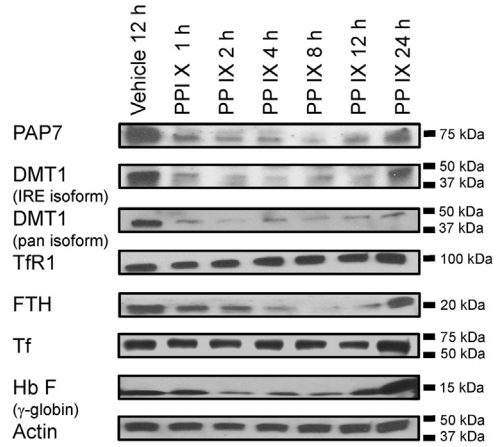

Fig. 2. Time course effects of PP IX on the protein expression level of peripheral-type benzodiazepine receptor (PBR)-associated protein 7 (PAP7), Divalent metal transporter 1 (DMT1), Transferrin receptor 1 (TfR1), Ferritn heavy chain (FTH), Transferrin (Tf), HbF (γ-globin) and actin. K562 cells grown overnight in serum free medium were treated with vehicle (DMSO) or PP IX 5 μM. At the indicated times (12 h for DMSO and 1, 2, 4, 8, 12 and 24 h for PP IX), cells were harvested for analysis by western blotting using the appropriate antibodies as detailed in the Methods to detect PAP7, DMT1 (IRE isoform), DMT1 (pan isoform), TfR1, FTH, Tf, HbF (γ-globin) and actin. The expression level of PAP7, DMT1 (IRE isoform) and DMT1 (pan isoform) were decreased after the treatment of PP IX. Shown is a representative experiment of three experiments.

greater than the PP IX concentration. Exogenous hemin also reduced the amount of heme resulting from exposure to 5 μM of PP IX to about the level seen with 2.5 μM of PP IX (Fig. 1A, S1A and B).

The intracellular concentrations of PP IX and heme were measured at various times after the addition of 5 μM of PP IX (Fig. 1B, S1C and D). There was a rapid increase of intracellular PP IX and heme by 1 h, followed by a slower increase of intracellular concentrations for both moieties to 12 h of exposure followed by a marked increase at 24 h. At all time points the intracellular levels of heme were about 20 fold more than the PPIX concentrations. Again, there was no effect of growth under low or high iron conditions (data not shown).

3.2. Time course effects of PP IX on the protein and mRNA expression level of peripheral-type benzodiazepine receptor (PBR)-associated protein 7 (PAP7), Divalent metal transporter 1 (DMT1), Transferrin receptor 1 (TfR1) and Ferritn heavy chain (FTH)

We initially examined the expression of PAP7 protein reasoning that if PP IX competed with PAP7 for binding to PBR that PAP7 levels might be affected. In fact, PP IX exposure decreased PAP7 protein levels in K562 cells grown under normal iron conditions (Fig. 2). The lower levels of PAP7 protein were apparent after one hour of exposure to PP IX and partially recovered at 24 h. The protein levels of DMT1 (Iron Responsive Element, IRE isoform) and DMT1 (pan isoform) were decreased by 1 h of exposure, remained low for 12 h, and exhibited some recovery at 24 h. FTH protein level fell more gradually with a complete recovery at 24 h. The protein level of TfR1 were increased without any effect on the amount of transferrin (Tf) bound to the cells. As expected PPIX, which is an inducer of erythroid differentiation in K562 cells, increased HbF (γ-globin) protein levels after 24 h of exposure, although at shorter exposure times the HbF protein levels were decreased. Similar changes in the protein levels of PAP7 and iron-related proteins were seen also in cells grown under high and low iron conditions (Fig. S2).

As PP IX had a rapid effect to decrease PAP7 protein expression, we examined the effects of PP IX on mRNA level of PAP7, DMT1 (IRE isoform), DMT1 (non-IRE isoform), TfR1 and FTH (Fig. 3). Measurements of PAP7 mRNA by real-time PCR showed that mRNA level of PAP7 was increased by 8 h and remained elevated for at least 24 h, after exposure to PP IX. PP IX decreased the mRNA for both the IRE and non-IRE isoforms of DMT1 for 24 h. In contrast, TfR1 and FTH mRNA levels increased significantly to peak at 12 h with FTH mRNA levels remaining elevated at 24 h. Similar patterns of response were observed in cells grown under low and high iron conditions (Fig. 3B and C).

3.3. The effect of PP IX compared to hemin on the protein and mRNA expression level of PAP7, DMT1, TfR1 and FTH

As exposure of the K562 cells to PP IX resulted in an increase of intracellular heme levels, we compared the effects of hemin on expression level of PAP7 and the various iron related proteins (Figs. 4 and 5). Hemin induced protein expression of HbF (γ-globin) as was expected of an inducer of erythropoietic differentiation (Fig. 4). Hemin also decreased TfR1 and increased FTH protein expression, changes consistent with hemin inducing an iron loaded phenotype. In contrast to PP IX, hemin affected neither PAP7 nor DMT1 protein expression, significantly. However, hemin reduced the PAP7 response to PP IX. Hemin suppressed the PP IX effect on DMT1 protein levels and modified the effect of PP IX on the expression level of mRNA and protein for TfR1 and FTH. The effects of hemin on mRNA levels were more complex. Hemin increased PAP7 mRNA levels by itself even in the presence of PP IX (Fig. 5). The effects of PP IX and hemin on mRNA were similar in the high-iron and low-iron condition (Fig. S4). Exposing the cells to high concentrations of iron (50 μM of FAC) did not alter PAP7 protein levels.

3.4. The effect of PP IX on C/EBPα and c-Myc expression

Because of the involvement of the transcription factors c-Myc and C/EBPα in expression of various genes involved in iron metabolism, we

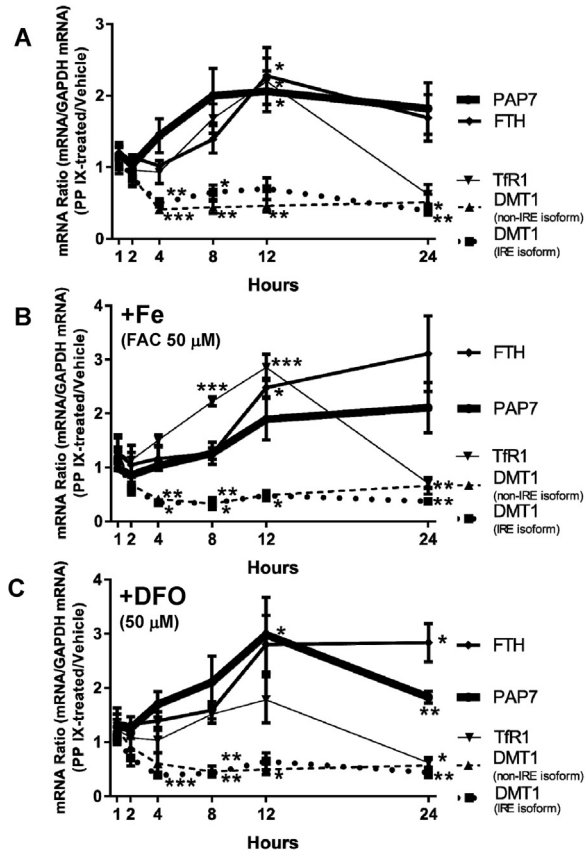

Fig. 3. The effect of PP IX on mRNA levels of PAP7, DMT1 (IRE isoform), DMT1 (non-IRE isoform), TfR1, FTH, as detected by quantitative real time PCR in different iron condition. K562 cells grown in serum free medium overnight in either (A) normal, (B) high iron (ferric ammonium citrate; FAC 50 μM) or (C) low iron (deferoxamine mesylate; DFO 50 μM) condition. Cells were treated either with vehicle (DMSO) or PP IX 5 μM and harvested at the indicated times (12 h for DMSO and 1, 2, 4, 8, 12 and 24 h for PP IX). Expression levels of mRNA were determined as detailed in the Methods with the amount of each mRNA expressed relative to the amount of GAPDH mRNA. Shown are means ± SEM of relative expression to vehicle alone at different time point of four experiments. An unpaired *t*-test was applied to the results with significant difference (*; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.0001$) from samples at 1 h-treatment of PP IX.

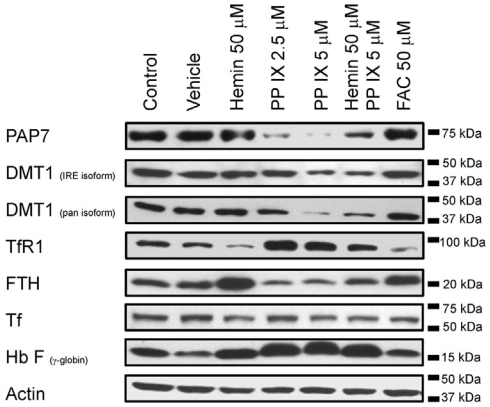

Fig. 4. The effect of PP IX and hemin on protein level of PAP7, DMT1 (IRE isoform), DMT1 (pan isoform), TfR1, FTH, Tf, HbF (γ-globin) and actin. K562 cells grown overnight in serum free medium were treated for 24 h with medium only (control), vehicle (DMSO), Hemin 50 μM, PP IX 2.5 μM, PP IX 5 μM, Hemin 50 μM + PP lX 5 μM, or Ferric Ammonium Citrate (FAC) 50 μM. Protein expression levels were determined by western blotting as detailed in the Methods for PAP7, DMT1 (IRE isoform), DMT1 (pan isoform), TfR1, FTH, Tf, HbF (γ-globin) and actin. Shown is a representative example of three experiments.

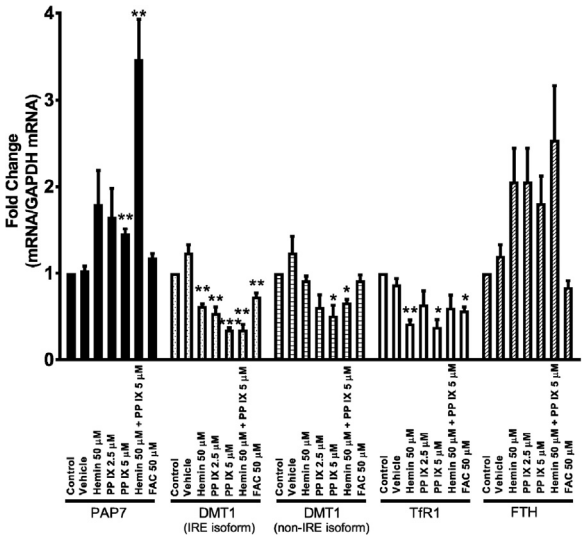

Fig. 5. The effect of PP IX and hemin on mRNA levels of PAP7, DMT1 (IRE isoform), DMT1 (non-IRE isoform), TfR1, FTH, as detected by quantitative real time PCR. K562 cells grown overnight in serum free medium were treated with medium only (control), Vehicle (DMSO), Hemin 50 μM, PP IX 2.5 μM, PP IX 5 μM, Hemin 50 μM + PP lX 5 μM, or Ferric Ammonium Citrate (FAC) 50 μM for 24 h. The expression levels of mRNA were determined by quantitative real time PCR for PAP7, DMT1 (IRE isoform), DMT1 (non-IRE isoform), TfR1 and FTH with the amount of each mRNA expressed relative to the amount of GAPDH mRNA. Shown are means ± SEM of three experiments. An unpaired *t*-test was applied to the results with significant differences (*; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$) from vehicle alone.

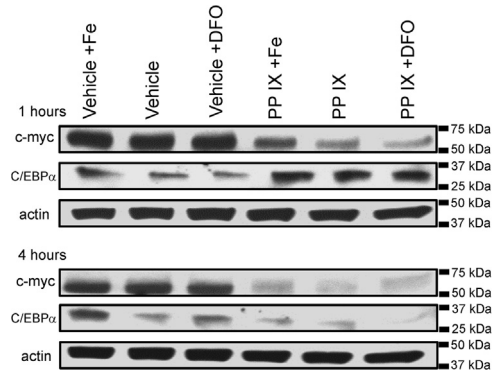

Fig. 6. The effect of PP IX on expression of c-Myc and C/EBPα protein. K562 cells grown overnight in serum free medium of either high iron (Ferric Ammonium Citrate; FAC 50 μM), normal iron, or low iron (deferoxamine mesylate; DFO 50 μM) condition. Cells were treated with vehicle (DMSO) or PP IX 5 μM. The protein expression levels were determined by Western blotting at 1 and 4 h after the addition of DMSO or PP IX. Shown is a representative experiment of three experiments.

examined the effects of PP IX on c-Myc and C/EBPα protein levels by western blot analysis (Fig. 6). While c-Myc levels were not affected by the iron status of the K562 cells, PPIX decreased c-Myc protein expression after 1 and 4 h of exposure. The response of C/EBPα protein level was increased of C/EBPα after 1 h of exposure to PPIX followed by the decrease after 4 h of exposure.

4. Discussion

We have shown that PAP7 affects the expression of DMT1 (IRE isoform) by a post-transcriptional mechanism and also affects cell proliferation [11]. PAP7 has been demonstrated to bind to the peripheral-type benzodiazepine receptor (PBR) and may be important for the transport of coproporphyrinogen from the cytosol into the mitochondria [3,20]. As PP IX has been identified as a ligand for PBR, we reasoned that exogenously added PP IX might compete for PAP7 for binding to PBR. As a consequence, the interaction of PAP7 with DMT1

may be altered and cellular iron metabolism may be affected, with any such changes giving insight into the role of PAP7.

Our first observation was that incubation of K562 cells with PP IX increased intracellular heme and PP IX concentrations. With the limitation of the number of time points and concentrations used in this study, the uptake of PP IX appeared to be triphasic. Heme/porphyrin transport proteins – Heme Carrier Protein 1 (HCP1), Feline Leukemia Virus subgroup C Cellular Receptor 1 (FLVCR1) and ATP-Binding Cassette subfamily G member 2 (ABCG2) – clearly affect flux of heme and porphyrin across the plasma membrane [21–23], while ATP-Binding Cassette subfamily B member 6 (ABCB6) has been defined as the transporter of porphyrins into the mitochondria [24]. Recent evidence suggests that HCP1 may be a folate transporter in intestinal cells although the role of HCP1 in erythroid precursors has to be defined [25,26]. Likewise, FLVCR and ABCG2 are important in efflux of heme but whether these transporters can also act in the reverse direction dependent on the heme or PP IX gradient is not established. The net result of the activities of these transporters and the potential for induction of activity with exposure to PP IX presumably is the cause of the observed pattern of uptake. It is of interest that most of the PP IX was converted to heme as the resulting heme concentrations were many fold higher than the PP IX concentrations and greater than that occurring when the cells were exposed to 50 μM of hemin alone. Whether the conversion of PP IX was enzymatic or non-enzymatic was not studied. In either event the source of the iron for the synthesis of heme was presumably intracellular iron pools and iron from Tf in the culture medium. As PP IX produced an iron deficiency phenotype with decreased FTH and increased TfR1 expression, then presumably the major source of iron was from intracellular pools. Effects seen with hemin supported this interpretation. Even though exogenous 50 μM hemin raised the intracellular levels of heme far less than PPIX, cells up-regulated FTH expression and down-regulated TfR1 expression, a phenotype consistent with iron overload.

There were clear differences in the consequences of the altered cellular iron levels created by hemin and the phenotype imposed by PP IX. Although both PP IX and hemin induced erythroid differentiation as measured by induction of HbF (γ-globin), PP IX down- regulated both PAP7 and DMT1 expression while hemin had no effect on the expression of either protein, strikingly. The decrease of PAP7 appeared to be at the level of protein turnover as PAP7 mRNA levels increased following hours of exposure to PP IX and PAP7 protein levels were restored at 24 h. DMT1 appears to be very stable with negligible turnover when expressed in LLC-PK_1 cells as the fully glycosylated form and with a half-life of about 8 h, when glycosylation is blocked [27]. In differentiating P19 embryonic carcinoma cells, the half-life of DMT1 protein was shown to be decreased from 15 h to 2–3 h by the proteosomal and lysosomal pathway [28]. Mutated DMT1 protein, which harbors the G185R in mk mice and Belgrade rats, has a markedly shortened survival and is degraded by proteasomes [29]. The rapid decrease in DMT1 protein is consistent with our hypothesis that PAP7 binding to DMT1 is important for DMT1 stability [11]. It remains to be seen if PAP7 blocks ubiquitination and proteasomal degradation or if PAP7 influences the glycosylation of DMT1 allowing for more rapid degradation. Although preliminary experiments with a combination of proteasome inhibitor and lysosome inhibitor prevented the decrease of FTH (Fig. S5), they did not prevent the rapid decrease of PAP7 or DMT1 protein (data not shown). Although we have examined the expression levels of DMT1 protein for pan isoform and +IRE isoform, 1B isoform, which is defined by the 5′ splicing, but not 3′ splicing, is shown to be critical by proteasomal degradation by Parkin [30]. Another ubiquitin-protein ligase, ndfip1 is also shown to be involved in DMT1 turnover [31,32]. The enzymatic activity of Ndfip1 against four DMT1 isoforms is not determined, however, 1 A/+IRE isoform is presumed to be degraded by Ndfip1 [30]. The role of Ndfip1 in PP IX-induced degradation of DMT1 still remains elucidated.

While clearly iron responsive proteins (IRPs) are essential in the regulation of expression of TfR1, FTH and DMT1 in erythroid differentiation [33], PP IX appears to modify the response with a transient rise and fall of TfR1 mRNA, an increase of FTH mRNA and a decrease of mRNA for both the IRE and non-IRE isoforms of DMT1. These changes would have been induced from altered intracellular iron pools imposed by PP IX to stimulate heme synthesis. These results were not consistent with the known activities of IRP as derived from intracellular iron pools. Recent studies have implicated that c-Myc and C/EBPα are important transcriptional factor in regulating various genes involved in cellular iron metabolism. For example, c-Myc has been described as controlling TfR1, DMT1, succinate dehydrogenase B, and frataxin levels [34], while C/EBPα is important in regulation of hepcidin [35], and DMT1 [36,37]. C/EBPα also affects iron metabolism indirectly by promoting granulopoiesis and is diminished in erythropoiesis [38]. Exposure of PP IX triggered to decrease the protein levels of C/EBPα, subsequently, down-regulated mRNA level of DMT1 IRE and non-IRE isoforms during erythroid differentiation. PPIX may have effects on regulation of iron-related mRNA expression beyond the canonical IRP system.

PBR has been widely accepted in the scientific community, renaming of the PBR to be a translocator protein (TSPO) was proposed. The reason for this proposal is that PBR has many ligands, being expressed in some cells of central nerves system, different subcellular localization, and incomplete understanding of the potential role in the transduction of cellular signaling pathways [7]. Recently, erythropoiesis and heme biosynthesis were shown to be maintained in TSPO knock out mice [39]. In glioma cell line, the accumulation of PP IX was observed after knock down of TSPO [40]. The function of PBR in cancer therapy and iron metabolism still remains elucidated.

In conclusion, we observed that PP IX decreased PAP7 and DMT1 in K562 cells. Further study may reveal the relation between utilization of labile iron pools and erythroid differentiation.

Acknowledgements

The study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Research Grants DK43785 (J Glass), the Carroll W. Feist Professorship, the Feist-Weiller Cancer Center, Louisiana University Health Sciences Center and partially supported by JSPS KAKENHI (15K08398) (Y Okazaki). The authors wish to thank Emiko Okazaki for technical assistance. The authors do not have any conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2017.02.007.

References

[1] D.R. Richardson, D.J. Lane, E.M. Becker, M.L. Huang, M. Whitnall, Y. Suryo Rahmanto, A.D. Sheftel, P. Ponka, Mitochondrial iron trafficking and the integration of iron metabolism between the mitochondrion and cytosol, Proc. Natl. Acad. Sci. USA 107 (2010) 10775–10782.

[2] S. Taketani, H. Kohno, T. Furukawa, R. Tokunaga, Involvement of peripheral-type benzodiazepine receptors in the intracellular transport of heme and porphyrins, J. Biochem. 117 (1995) 875–880.

[3] A. Verma, J.S. Nye, S.H. Snyder, Porphyrins are endogenous ligands for the mitochondrial (peripheral-type) benzodiazepine receptor, Proc. Natl. Acad. Sci. USA 84 (1987) 2256–2260.

[4] G. Wendler, P. Lindemann, J.J. Lacapere, V. Papadopoulos, Protoporphyrin IX binding and transport by recombinant mouse PBR, Biochem. Biophys. Res. Commun. 311 (2003) 847–852.

[5] V. Papadopoulos, In search of the function of the peripheral-type benzodiazepine receptor, Endocr. Res. 30 (2004) 677–684.

[6] J. Fan, J. Liu, M. Culty, V. Papadopoulos, Acyl-coenzyme A binding domain containing 3 (ACBD3; PAP7; GCP60): an emerging signaling molecule, Prog. Lipid Res. 49 (2010) 218–234.

[7] V. Papadopoulos, M. Baraldi, T.R. Guilarte, T.B. Knudsen, J.J. Lacapere, P. Lindemann, M.D. Norenberg, D. Nutt, A. Weizman, M.R. Zhang, M. Gavish,

Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function, Trends Pharmacol. Sci. 27 (2006) 402–409.
[8] M. Ishizuka, F. Abe, Y. Sano, K. Takahashi, K. Inoue, M. Nakajima, T. Kohda, N. Komatsu, S. Ogura, T. Tanaka, Novel development of 5-aminolevurinic acid (ALA) in cancer diagnoses and therapy, Int. Immunopharmacol. 11 (2011) 358–365.
[9] K.T. Moesta, B. Ebert, T. Handke, D. Nolte, C. Nowak, W.E. Haensch, R.K. Pandey, T.J. Dougherty, H. Rinneberg, P.M. Schlag, Protoporphyrin IX occurs naturally in colorectal cancers and their metastases, Cancer Res. 61 (2001) 991–999.
[10] L. Teng, M. Nakada, S.G. Zhao, Y. Endo, N. Furuyama, E. Nambu, I.V. Pyko, Y. Hayashi, J.I. Hamada, Silencing of ferrochelatase enhances 5-aminolevulinic acid-based fluorescence and photodynamic therapy efficacy, Br. J. Cancer 104 (2011) 798–807.
[11] Y. Okazaki, Y. Ma, M. Yeh, H. Yin, Z. Li, K.Y. Yeh, J. Glass, DMT1 (IRE) expression in intestinal and erythroid cells is regulated by peripheral benzodiazepine receptor-associated protein 7, Am. J. Physiol. Gastrointest. Liver Physiol. 302 (2012) G1180–G1190.
[12] T. Lambe, R.J. Simpson, S. Dawson, T. Bouriez-Jones, T.L. Crockford, M. Lepherd, G.O. Latunde-Dada, H. Robinson, K.B. Raja, D.R. Campagna, G. Villarreal Jr., J.C. Ellory, C.C. Goodnow, M.D. Fleming, A.T. McKie, R.J. Cornall, Identification of a Steap3 endosomal targeting motif essential for normal iron metabolism, Blood 113 (2009) 1805–1808.
[13] D.J. Lane, A.M. Merlot, M.L. Huang, D.H. Bae, P.J. Jansson, S. Sahni, D.S. Kalinowski, D.R. Richardson, Cellular iron uptake, trafficking and metabolism: Key molecules and mechanisms and their roles in disease, Biochim. Biophys. Acta 1853 (2015) 1130–1144.
[14] Y. Ma, M. Yeh, K.Y. Yeh, J. Glass, V. Iron Imports, Transport of iron through the intestinal epithelium, Am. J. Physiol. Gastrointest. Liver Physiol. 290 (2006) G417–G422.
[15] J.H. Cheah, S.F. Kim, L.D. Hester, K.W. Clancy, S.E. Patterson 3rd, V. Papadopoulos, S.H. Snyder, NMDA receptor-nitric oxide transmission mediates neuronal iron homeostasis via the GTPase Dexras1, Neuron 51 (2006) 431–440.
[16] M.A. Ostuni, G. Peranzi, R.A. Ducroc, M. Fasseu, B. Vidic, J. Dumont, V. Papadopoulos, J.J. Lacapere, Distribution, pharmacological characterization and function of the 18 kDa translocator protein in rat small intestine, Biol. Cell. 101 (2009) 573–586.
[17] E.M. Coccia, E. Perrotti, E. Stellacci, R. Orsatti, N. Del Russo, G. Marziali, U. Testa, A. Battistini, Regulation of expression of ferritin H-chain and transferrin receptor by protoporphyrin IX, Eur. J. Biochem. 250 (1997) 764–772.
[18] O. Nakajima, Y. Hashimoto, S. Iwasaki, Erythroid differentiation-inducing activity of protoporphyrin IX and its analogs on human leukemia K562 cell line, Biochem. Biophys. Res. Commun. 198 (1994) 720–727.
[19] K.Y. Yeh, M. Yeh, J.A. Watkins, J. Rodriguez-Paris, J. Glass, Dietary iron induces rapid changes in rat intestinal divalent metal transporter expression, Am. J. Physiol. Gastrointest. Liver Physiol. 279 (2000) G1070–G1079.
[20] S. Taketani, H. Kohno, M. Okuda, T. Furukawa, R. Tokunaga, Induction of peripheral-type benzodiazepine receptors during differentiation of mouse erythroleukemia cells. A possible involvement of these receptors in heme biosynthesis, J. Biol. Chem. 269 (1994) 7527–7531.
[21] J.W. Jonker, M. Buitelaar, E. Wagenaar, M.A. Van Der Valk, G.L. Scheffer, R.J. Scheper, T. Plosch, F. Kuipers, R.P. Elferink, H. Rosing, J.H. Beijnen, A.H. Schinkel, The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria, Proc. Natl. Acad. Sci. USA 99 (2002) 15649–15654.
[22] S.B. Keel, R.T. Doty, Z. Yang, J.G. Quigley, J. Chen, S. Knoblaugh, P.D. Kingsley, I. De Domenico, M.B. Vaughn, J. Kaplan, J. Palis, J.L. Abkowitz, A heme export protein is required for red blood cell differentiation and iron homeostasis, Science 319 (2008) 825–828.
[23] M. Shayeghi, G.O. Latunde-Dada, J.S. Oakhill, A.H. Laftah, K. Takeuchi, N. Halliday, Y. Khan, A. Warley, F.E. McCann, R.C. Hider, D.M. Frazer, G.J. Anderson, C.D. Vulpe, R.J. Simpson, A.T. McKie, Identification of an intestinal heme transporter, Cell 122 (2005) 789–801.
[24] P.C. Krishnamurthy, G. Du, Y. Fukuda, D. Sun, J. Sampath, K.E. Mercer, J. Wang, B. Sosa-Pineda, K.G. Murti, J.D. Schuetz, Identification of a mammalian mitochondrial porphyrin transporter, Nature 443 (2006) 586–589.
[25] S. Le Blanc, M.D. Garrick, M. Arredondo, Heme carrier protein 1 transports heme and is involved in heme-Fe metabolism, Am. J. Physiol. Cell Physiol. 302 (2012) C1780–C1785.
[26] A. Qiu, M. Jansen, A. Sakaris, S.H. Min, S. Chattopadhyay, E. Tsai, C. Sandoval, R. Zhao, M.H. Akabas, I.D. Goldman, Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption, Cell 127 (2006) 917–928.
[27] S. Lam-Yuk-Tseung, P. Gros, Distinct targeting and recycling properties of two isoforms of the iron transporter DMT1 (NRAMP2, Slc11A2), Biochemistry 45 (2006) 2294–2301.
[28] P.N. Paradkar, J.A. Roth, Post-translational and transcriptional regulation of DMT1 during P19 embryonic carcinoma cell differentiation by retinoic acid, Biochem. J. 394 (2006) 173–183.
[29] N. Touret, N. Martin-Orozco, P. Paroutis, W. Furuya, S. Lam-Yuk-Tseung, J. Forbes, P. Gros, S. Grinstein, Molecular and cellular mechanisms underlying iron transport deficiency in microcytic anemia, Blood 104 (2004) 1526–1533.
[30] M.D. Garrick, L. Zhao, J.A. Roth, H. Jiang, J. Feng, N.J. Foot, H. Dalton, S. Kumar, L.M. Garrick, Isoform specific regulation of divalent metal (ion) transporter (DMT1) by proteasomal degradation, Biometals 25 (2012) 787–793.
[31] N.J. Foot, H.E. Dalton, L.M. Shearwin-Whyatt, L. Dorstyn, S.S. Tan, B. Yang, S. Kumar, Regulation of the divalent metal ion transporter DMT1 and iron homeostasis by a ubiquitin-dependent mechanism involving Ndfips and WWP2, Blood 112 (2008) 4268–4275.
[32] N.J. Foot, Y.A. Leong, L.E. Dorstyn, H.E. Dalton, K. Ho, L. Zhao, M.D. Garrick, B. Yang, D. Hiwase, S. Kumar, Ndfip1-deficient mice have impaired DMT1 regulation and iron homeostasis, Blood 117 (2011) 638–646.
[33] J. Kato, M. Kobune, S. Ohkubo, K. Fujikawa, M. Tanaka, R. Takimoto, K. Takada, D. Takahari, Y. Kawano, Y. Kohgo, Y. Niitsu, Iron/IRP-1-dependent regulation of mRNA expression for transferrin receptor, DMT1 and ferritin during human erythroid differentiation, Exp. Hematol. 35 (2007) 879–887.
[34] K.A. O'Donnell, D. Yu, K.I. Zeller, J.W. Kim, F. Racke, A. Thomas-Tikhonenko, C.V. Dang, Activation of transferrin receptor 1 by c-Myc enhances cellular proliferation and tumorigenesis, Mol. Cell. Biol. 26 (2006) 2373–2386.
[35] B. Courselaud, C. Pigeon, Y. Inoue, J. Inoue, F.J. Gonzalez, P. Leroyer, D. Gilot, K. Boudjema, C. Guguen-Guillouzo, P. Brissot, O. Loreal, G. Ilyin, C/EBPalpha regulates hepatic transcription of hepcidin, an antimicrobial peptide and regulator of iron metabolism. Cross-talk between C/EBP pathway and iron metabolism, J. Biol. Chem. 277 (2002) 41163–41170.
[36] P.L. Lee, T. Gelbart, C. West, C. Halloran, E. Beutler, The human Nramp2 gene: characterization of the gene structure, alternative splicing, promoter region and polymorphisms, Blood Cells Mol. Dis. 24 (1998) 199–215.
[37] P.N. Paradkar, J.A. Roth, Expression of the 1B isoforms of divalent metal transporter (DMT1) is regulated by interaction of NF-Y with a CCAAT-box element near the transcription start site, J. Cell. Physiol. 211 (2007) 183–188.
[38] H.C. Suh, J. Gooya, K. Renn, A.D. Friedman, P.F. Johnson, J.R. Keller, C/EBPalpha determines hematopoietic cell fate in multipotential progenitor cells by inhibiting erythroid differentiation and inducing myeloid differentiation, Blood 107 (2006) 4308–4316.
[39] A.H. Zhao, L.N. Tu, C. Mukai, M.P. Sirivelu, V.V. Pillai, K. Morohaku, R. Cohen, V. Selvaraj, Mitochondrial translocator protein (TSPO) function is not essential for heme biosynthesis, J. Biol. Chem. 291 (2016) 1591–1603.
[40] S. Zeno, L. Veenman, Y. Katz, J. Bode, M. Gavish, M. Zaaroor, The 18 kDa mitochondrial translocator protein (TSPO) prevents accumulation of protoporphyrin IX. involvement of reactive oxygen species (ROS), Curr. Mol. Med. 12 (2012) 494–501.