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

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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

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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 horse-radish 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

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