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Research Article

Cell Survival under Stress Is Enhanced by a Mitochondrial ATP-Binding Cassette Transporter That Regulates Hemoproteins

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Abstract

The ATP-binding cassette (ABC) transporter ABCB6 localizes to the mitochondria, where it imports porphyrins and up-regulates *de novo* porphyrin synthesis. If ABCB6 also increases the intracellular heme concentration, it may broadly affect the regulation and physiology of cellular hemoproteins. We tested whether the ability of ABCB6 to accelerate *de novo* porphyrin biosynthesis alters mitochondrial and extramitochondrial heme levels. ABCB6 overexpression increased the quantity of cytosolic heme but did not affect mitochondrial heme levels. We then tested whether the increased extramitochondrial heme would increase the concentration and/or activity of cellular hemoproteins (hemoglobin, catalase, and cytochrome *c* oxidase). ABCB6 overexpression increased the activity and quantity of hemoproteins found in several subcellular compartments, and reduction of ABCB6 function (by small interfering RNA or knockout) reversed these findings. In complementary studies, suppression of ABCB6 expression sensitized cells to stress induced by peroxide and cyanide, whereas overexpression of ABCB6 protected against both stressors. Our findings show that the ability of ABCB6 to increase cytosolic heme levels produces phenotypic changes in hemoproteins that protect cells from certain stresses. Collectively, these findings have implications for the health and survival of both normal and abnormal cells, which rely on heme for multiple cellular processes. [Cancer Res 2009; 69(13):5560–7]

Introduction

ATP-binding cassette (ABC) transporters use ATP to facilitate transmembrane movement of a variety of structurally diverse compounds. We recently characterized the mitochondrial ABC transporter ABCB6 (1) and found that ABCB6 binds porphyrins, including heme. We showed that ABCB6 overexpression increases *de novo* porphyrin biosynthesis and the mitochondrial concentration of protoporphyrin IX (PPIX), the penultimate precursor of heme. However, we did not assess changes in heme concentration.

It is likely that ferrochelatase limits the rate of heme biosynthesis in the mitochondria by limiting the conversion of PPIX to heme (2, 3). If this is not the case, then the mitochondrial heme concentration may increase in parallel with ABCB6-mediated increases in PPIX concentration. However, the mechanism of heme transit from the mitochondria to the cytosol is unknown, and increased mitochondrial heme does not necessarily predict increased cytosolic heme. Because the heme components iron and PPIX are toxic to cells (iron generates hydroxyl radicals by Fenton-based reactions and PPIX catalyzes light-dependent generation of oxygen radicals), it is important to know whether increased ABCB6 expression results in greater heme biosynthesis and accumulation in cytosol and/or mitochondria.

Movement of heme from the mitochondria to the cytosol requires protein synthesis (4–7), and movement of heme through the cytosol is likely to require chaperones. Because the translation or assembly of some hemoproteins [e.g., globin (8), catalase, and cytochrome *c* oxidase (9–13)] requires heme, the concentration of these proteins may depend on the availability of cytosolic heme. However, increased cellular heme is not always associated with an increase in hemoproteins. For example, overexpression of the mitochondrial ABC transporter ABCB7 increases heme levels but has no effect on the level of catalase (14), suggesting that processes downstream of ferrochelatase limit the rate of hemoprotein formation.

Here, we show that ABCB6 overexpression increases the intracellular concentration of heme and hemoglobin via *de novo* porphyrin synthesis. ABCB6 also increases the activity or concentration of hemoproteins that protect against certain stresses. These results establish the interrelation of ABCB6 function, heme, hemoproteins, and cell survival.

Materials and Methods

Cell culture. K562 myelogenous leukemia cells transduced with either empty vector (MSCV) or vector encoding ABCB6 were maintained, as previously described (1). Mouse embryonic fibroblasts (MEF) were obtained from $Abcb6^{-/-}$ (the development and characterization of $Abcb6^{-/-}$ animals will be described elsewhere) and $Abcb6^{+/+}$ embryos (E13.5) sacrificed by decapitation, washed with PBS, and trypsinized. Cells were disaggregated by passage through a 21-gauge needle and maintained at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mmol/L glutamine (Life Technologies), 50 mg/mL penicillin/50 units/mL streptomycin (Life Technologies), MEM nonessential amino acids (Life Technologies), and 55 μmol/L β-mercaptoethanol.

Isolation and purification of mitochondria. Cells were pelleted in 1× Hanks buffered saline solution (Life Technologies), resuspended in buffer A [10 mmol/L NaCl, 1.5 MgCl$_2$, 10 mmol/L Tris (pH 7.4)] containing 1× protease inhibitor cocktail (Roche), swollen on ice, and disrupted with a type B Dounce homogenizer. Buffer B [525 mmol/L mannitol, 175 mmol/L sucrose, 12.5 mmol/L Tris (pH 7.4), and 2.5 mmol/L EDTA] was added in a ratio of 4:10 homogenate/buffer B. The supernatant was collected after centrifugation at 1,300 × *g* for 10 min. This step was repeated until no pellet was visible. The remaining supernatant was centrifuged at 14,000 × *g* for 15 min to pellet mitochondria. The crude mitochondria were purified from the endoplasmic reticulum (ER) as previously described (14).

Enzyme and hemoglobin assays. PPIX concentration was measured by fluorescence-activated cell sorting (1). Lactate dehydrogenase (LDH)

activity was measured as reduction of the tetrazolium salt substrate to the soluble formazan in the presence of NADH by using the LDH kit from Biomedical Research Service Center (Buffalo, NY). Catalase was assayed by using a kit from BRSC. Superoxide dismutase activity was determined by using a kit (Fluka) according to the manufacturer's instructions. Succinate dehydrogenase activity was assayed by the reduction of 2-14-iodophenyl 3-(4-nitrophenyl) 5-phenyl tetrazolium chloride from the tetrazolium salt to the formazan. After ethyl acetate extraction, absorbance was read at 490 nm and concentration was determined using a molar extinction coefficient of 11.5 mmol/L^{-1} cm^{-1}. Cytochrome *c* oxidase activity was measured as the oxidation of cytochrome *c* at 550 nm. Hemoglobin concentration was measured spectrophotometrically by absorbance at 414 nm.

High-performance liquid chromatography assay of cellular heme. Extraction and high-performance liquid chromatography (HPLC) analysis of heme was performed essentially as described (15). Briefly, heme was extracted from disrupted cell lysates with acidified acetone (2.5% HCl). Extracts were clarified by centrifugation and analyzed by reverse-phase HPLC using a 4.6-μm × 150-mm C18 column (heme b retention time was 16 min at a flow rate of 1 mL/min). The acetonitrile elution gradient increased from 30% to 60% acetonitrile over the first 5 min and from 60% to 90% over the next 35 min. TCA (0.05%) was included in all mobile phases. Absorbance was read at 400 nm; the range of linear detection of heme was 160 nmol/L to 5 μmol/L.

Protein synthesis inhibition. K562 cells were seeded at 2×10^5/mL in DMEM with 10% supplemented FBS (Hyclone), 50 mg/mL penicillin/50 units/mL streptomycin (Life Technologies), and 2 mmol/L L-glutamine (Life Technologies). Cycloheximide (50 μg/mL) was added to inhibit protein synthesis for the indicated time intervals.

Cytotoxicity. K562 or MEF cells were seeded as described above, treated with the indicated concentrations of H_2O_2 or KCN for 4 h, washed with 1× Hanks buffered saline solution, and assessed for viability by trypan blue dye exclusion.

***De novo* porphyrin synthesis.** The *de novo* synthesis of porphyrins was measured by analyzing incorporation of radiolabeled [^{14}C]glycine, as previously described (1).

Results

ABCB6 overexpression increases the PPIX, heme, and hemoglobin content of K562 cells. A recent report suggested that ABCB6 localizes to the plasma membrane, as well as to the mitochondria (16). Therefore, we first wished to confirm the localization of ABCB6 in K562 cells retrovirally transduced with empty vector or *ABCB6*. Our previous studies showed mitochondrial, but not plasma membrane, localization of ABCB6 in these cells (1). To rule out possible ER contamination, we separated ER and mitochondrial fractions by limited proteolysis, as previously described (17). We then separated the soluble proteolyzed fragments from the pellet by centrifugation. ABCB6 colocalized with the mitochondrial marker VDAC in the pellet, but not with the ER marker Grp94 in the supernatant (Fig. 1*A*). Therefore, ABCB6 localizes to the mitochondria in K562 cells and not to the ER.

K562 cells expressing ABCB6 showed increased PPIX biosynthesis as reflected by increased PPIX fluorescence, whereas those
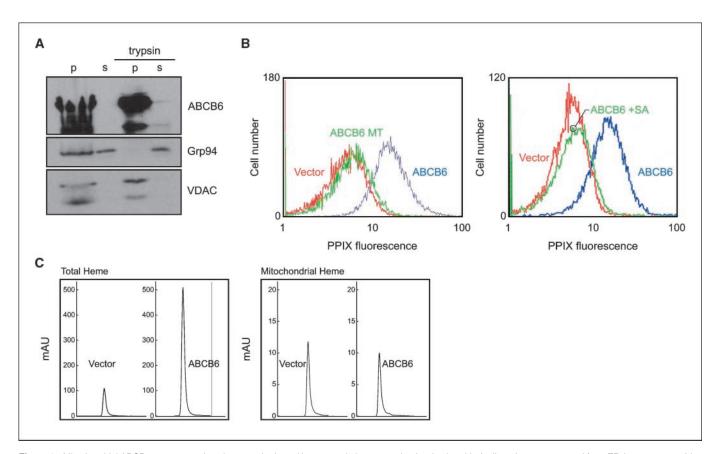

Figure 1. Mitochondrial ABCB6 overexpression alters porphyrin and hemoprotein homeostasis. *A,* mitochondria (pellet, *p*) were separated from ER (supernatant, *s*) by limited tryptic digestion. The fractions were separated by PAGE and probed with antibodies to the indicated proteins. The reduced quantity of the proteins is likely due to proteolysis during trypsin treatment. *B,* PPIX concentration is elevated in K562 cells overexpressing ABCB6, but not in cells expressing a nonfunctional mutant (*MT*) ABCB6 (1). The PPIX level is reduced by treatment with 200 μmol/L succinylacetone (*SA*), which does not affect cell viability as assessed by trypan blue dye exclusion. *C,* total cellular heme, but not mitochondrial heme, is increased in ABCB6-overexpressing cells as determined by HPLC using heme b as a standard.
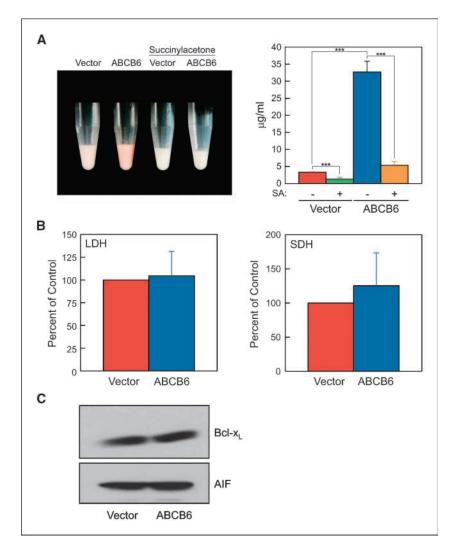
Figure 2. ABCB6 overexpression increases cellular hemoglobin concentration. *A,* the red coloration (*left*) and the spectrophotometrically measured hemoglobin concentration (*right*) in ABCB6-overexpressing and control cells are reduced by succinylacetone (*SA*) treatment. *Columns,* mean from three experiments; *bars,* SD. ***, $P < 0.001$. *B,* mean cytosolic LDH and mitochondrial succinate dehydrogenase activity are not changed by ABCB6 overexpression ($n = 3$). *C,* the quantity of Bcl-X_L and apoptosis-inducing factor (*AIF*) protein did not differ in ABCB6-overexpressing cells and vector-control cells.

expressing the nonfunctional Walker A mutant (1) ABCB6 did not (Fig. 1*B, left*). Addition of succinylacetone, a specific inhibitor of ALA dehydratase, the second enzyme in *de novo* porphyrin synthesis, reduced the level of PPIX in ABCB6-expressing cells compared with that in vector-control cells (Fig. 1*B, right*; ref. 1). HPLC analysis then showed intracellular heme concentration to be more than four times as great in ABCB6-overexpressing cells compared with that in vector-control cells (Fig. 1*C, left*). Unexpectedly, the heme content of purified mitochondria was almost identical in ABCB6-expressing and vector-control cells (Fig. 1*C, right*). Because derepression of globin translation by the heme-regulated inhibitor requires cytosolic heme (8), we examined whether ABCB6 expression increased hemoglobin concentration. ABCB6-expressing cells showed a dramatically greater hemoglobin content than did vector-control cells, unless they were treated with succinylacetone (Fig. 2*A*). Therefore, the greater hemoglobin content of ABCB6-expressing cells reflected greater *de novo* porphyrin biosynthesis. (Succinylacetone treatment reduced hemoglobin content similarly in vector-control (61%) and ABCB6-expressing (84%) cells; Supplementary Fig. S1).

The increased heme in ABCB6-expressing cells did not alter the expression of either mitochondrial or cytosolic proteins; the activity of cytosolic LDH and mitochondrial succinate dehydroge-

nase (an iron-sulfur cluster protein containing a heme-associated domain of unknown function) was unchanged by ABCB6 overexpression (Fig. 2*B*).

The BH$_3$ protein Bcl-X_L reportedly plays a role in regulating heme synthesis (18). However, the increased heme and hemoglobin content of ABCB6-overexpressing cells was not accompanied by a change in Bcl-X_L expression (Fig. 2*C*). Furthermore, ABCB6 overexpression does not affect the concentration of mitochondrial adenine nucleotide translocator (see Supplementary Fig. S2), a mitochondrial inner membrane transporter with a recently reported novel role in heme transport (19).

ABCB6 overexpression increases catalase protein and activity. Catalase is assembled in the peroxisomes (4), where the catalase apoprotein is combined with imported heme (4). The quantity and activity of catalase were strongly elevated (7-fold and 12-fold, respectively) in ABCB6-overexpressing cells (Fig. 3*A*). Superoxide dismutase activity increased only slightly with ABCB6 overexpression (data not shown); therefore, the increased catalase expression was not induced by a superoxide dismutase-mediated increase in hydrogen peroxide. Because catalase detoxifies hydrogen peroxide, we compared the hydrogen peroxide sensitivity of ABCB6-overexpressing cells and vector cells. ABCB6-over-expressing cells were four times as resistant to hydrogen peroxide
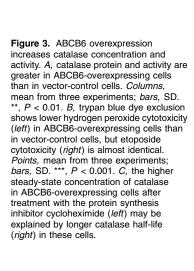
(estimated IC_{50}, 8 mmol/L versus <2 mmol/L; Fig. 3B, *left*). However, vector and ABCB6 cells were equally sensitive to etoposide (Fig. 3B, *right*); therefore, this finding did not reflect general resistance to cytotoxic agents. Furthermore, glutathione concentration was not significantly affected by ABCB6 overexpression (data not shown). The stability of catalase is related to heme concentration (9, 10); therefore, we compared the turnover of catalase in ABCB6 cells and vector cells treated for various intervals with the protein synthesis inhibitor cycloheximide (Fig. 3C). Immunoreactive catalase declined rapidly in vector cells, but not in ABCB6-overexpressing cells (estimated half-life, 8.7 h versus 88.5 h), a finding consistent with heme-mediated stabilization of catalase (9–11).
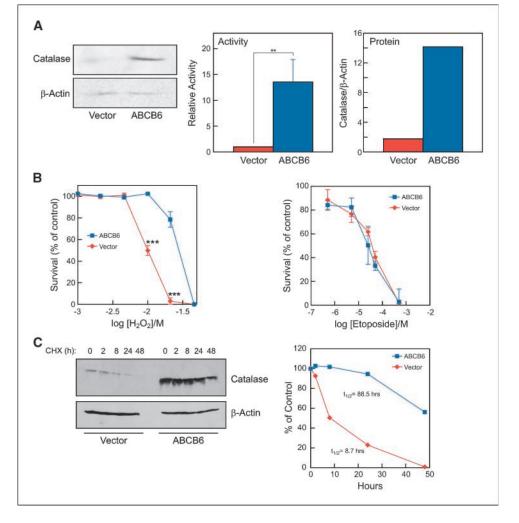
Catalase up-regulation depends on ABCB6 expression and *de novo* porphyrin synthesis. We previously showed that, when ABCB6 expression is reduced by *ABCB6* small interfering RNA (siRNA), *de novo* porphyrin synthesis is also reduced (1). To determine whether ABCB6 mediates catalase up-regulation, we treated ABCB6-expressing cells with *ABCB6* siRNA or scrambled siRNA for 48 hours. The *ABCB6* siRNA substantially reduced ABCB6 protein and significantly (40%; $P < 0.05$) reduced mean catalase activity (Fig. 4A and B). This result is consistent with the estimated half-life of ABCB6 (~ 24 hours) and catalase (~ 88 hours; Fig. 3C) in ABCB6-overexpressing cells. We next tested whether

siRNA knockdown of ABCB6 altered sensitivity to hydrogen peroxide. Cells ectopically expressing ABCB6 were significantly more resistant than vector cells to high concentrations of hydrogen peroxide but transfection with *ABCB6* siRNA reduced resistance to a level near that of vector cells (Fig. 4C). We attribute the differences in cell survival between this experiment and that in Fig. 3B to the conditions required for transfection of the siRNAs.

Because the levels of porphyrin biosynthesis and ABCB6 expression are directly linked (1), we next treated ABCB6-expressing and vector cells with succinylacetone to inhibit porphyrin biosynthesis and measured catalase activity. Catalase activity was reduced 70% in vector cells, but only 30% in ABCB6-overexpressing cells (Fig. 4B); this finding is consistent with greater heme concentration, which facilitates catalase stabilization (9, 11), in ABCB6-overexpressing cells.

ABCB6 expression increases cytochrome *c* oxidase activity and cyanide resistance. Mitochondrial cytochrome *c* oxidase comprises 13 subunits, and its function depends on the synthesis and availability of heme (12, 20–22). Chemical inhibition of heme synthesis and genetic defects in *de novo* heme synthesis reduce activity by interfering with subunit assembly (23–25). Mitochondrial cytochrome *c* oxidase activity was >4-fold greater in ABCB6-overexpressing cells than in vector cells (Fig. 5A, *left*). Notably, the expression of two mitochondrial-encoded subunits and one

Figure 3. ABCB6 overexpression increases catalase concentration and activity. *A,* catalase protein and activity are greater in ABCB6-overexpressing cells than in vector-control cells. *Columns,* mean from three experiments; *bars,* SD. **, $P < 0.01$. *B,* trypan blue dye exclusion shows lower hydrogen peroxide cytotoxicity (*left*) in ABCB6-overexpressing cells than in vector-control cells, but etoposide cytotoxicity (*right*) is almost identical. *Points,* mean from three experiments; *bars,* SD. ***, $P < 0.001$. *C,* the higher steady-state concentration of catalase in ABCB6-overexpressing cells after treatment with the protein synthesis inhibitor cycloheximide (*left*) may be explained by longer catalase half-life (*right*) in these cells.

nuclear-encoded subunit was unaltered by ABCB6 expression (Supplementary Fig. S3). Cells overexpressing ABCB6 were also more resistant to cyanide toxicity (Fig. 5*A, right*), a finding consistent with elevated cytochrome *c* oxidase activity.

Insufficient mitochondria were isolated to allow measurement of cytochrome *c* oxidase activity after siRNA transfection; therefore, we used mitochondria isolated from $Abcb6^{-/-}$ MEFs for this comparison. Because the basal cytochrome *c* oxidase activity differed by a factor of ~70 in MEFs and K562 cells, the reduction in cytochrome *c* oxidase activity in $Abcb6^{-/-}$ MEFs was more variable than anticipated (Fig. 5*B, left*) but was approximately half that in $Abcb6^{+/+}$ MEFs, consistent with the 30% lower rate of *de novo* porphyrin biosynthesis observed in $Abcb6^{-/-}$ MEFs (Supplementary Fig. S4). The reduced cytochrome *c* oxidase activity in $Abcb6^{-/-}$ MEFs paralleled their greater sensitivity to cyanide (Fig. 5*B, right*). To confirm that *de novo* porphyrin synthesis is the main pathway by which ABCB6 increases cytochrome *c* oxidase activity, we treated K562 cells with succinylacetone. Cytochrome *c* oxidase activity was reduced similarly in vector cells (85%) and ABCB6-expressing cells (94%; Fig. 5*C*). Collectively, these findings indicate that ABCB6 expression regulates cytochrome *c* oxidase activity by activating *de novo* heme biosynthesis.

Discussion

We previously showed that ABCB6 is primarily localized in the outer mitochondrial membrane and regulates *de novo* porphyrin biosynthesis, i.e., increased ABCB6 expression leads to increased PPIX concentration (1). Here, we extended this to show that ABCB6 is not localized to the ER in K562 cells. We further show that ABCB6 overexpression increases extramitochondrial (but not mitochondrial) heme concentration 4–5-fold. This result implies that neither conversion of PPIX to heme (via ferrochelatase) nor mitochondrial heme efflux limits the accumulation of extramito- chondrial heme when ABCB6 is overexpressed. Given that extramitochondrial heme is required for activation of globin synthesis (8), our findings show that ABCB6 overexpression increases hemoglobin levels. ABCB6 overexpression also increased the function, quantity, and/or activity of two other hemoproteins, catalase, and cytochrome *c* oxidase. These findings show that ABCB6 is important in regulating not only heme synthesis and concentration but also the formation of hemoproteins.

Our findings suggest that, by modulating intracellular heme concentration, ABCB6 plays a primary role in regulating hemopro- teins (1). For example, catalase assembly within the peroxisomes requires heme (4) and both heme levels and catalase activity were reduced in cells treated with succinylacetone (1) or with *ABCB6* siRNA. Although *de novo* porphyrin biosynthesis and catalase activity were reduced to a different extent, this discrepancy is likely to reflect the time required to deplete the heme pool and the relation between catalase stability and heme concentration (9, 11). The latter factor is illustrated by the long half-life of catalase (88.5 hours) and the diminished ability of succinylacetone treatment (2 days) to reduce catalase activity in ABCB6-expressing cells. The 40% reduction of catalase activity by *ABCB6* siRNA was sufficient to sensitize the cells to high concentrations of hydrogen
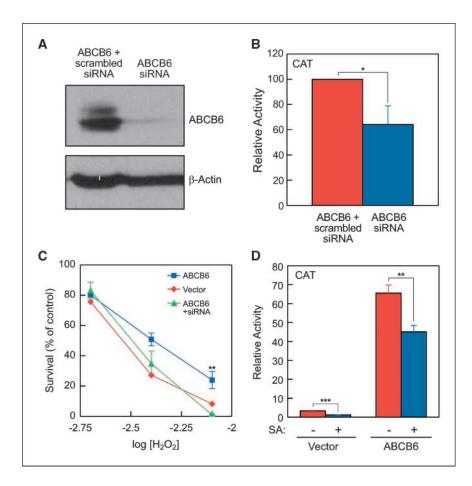

Figure 4. Silencing of *ABCB6* reduces catalase activity. *A,* the quantity of ABCB6 protein is lower at 72 h after treatment with *ABCB6* siRNA than 72 h after treatment with the scrambled siRNA control (1). *B,* in K562 cell extracts prepared 72 h after siRNA transfection, catalase activity normalized to the scrambled siRNA (100% activity) is significantly reduced in cells treated with *ABCB6* siRNA. *Columns,* mean from three experiments; *bars,* SD. *C,* siRNA reduction of ABCB6 sensitizes cells to hydrogen peroxide. *Points,* mean from three experiments; *bars,* SD. *D,* catalase activity is much lower in vector-control than in ABCB6-overexpressing K562 cells after treatment with succinylacetone (*SA*). *Columns,* mean from four experiments; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
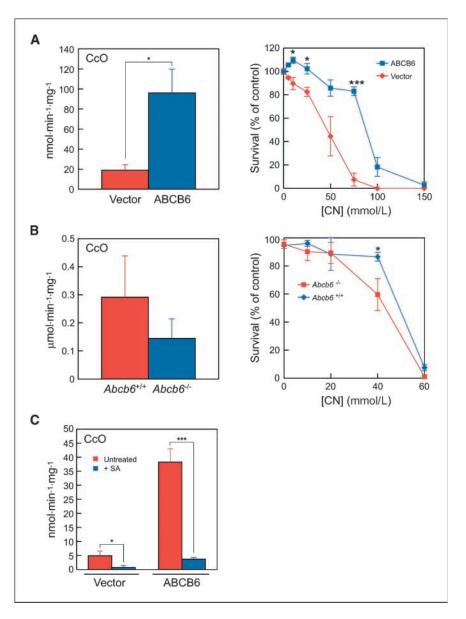
Figure 5. ABCB6 increases cytochrome *c* oxidase activity and cyanide resistance. *A,* ABCB6 overexpression increases cytochrome *c* oxidase (*CcO*) activity 5-fold (*left*). *Columns,* mean from four experiments; *bars,* SE. *, $P < 0.05$; ***, $P < 0.001$. ABCB6 overexpression induces strong resistance to cyanide (*CN*; a cytochrome *c* oxidase inhibitor), as measured by trypan blue dye exclusion (*right*). *Points,* mean from three experiments; *bars,* SD. *B,* mitochondrial CcO activity (*left*) and cyanide toxicity (*right*) in *Abcb6*$^{+/+}$ and *Abcb6*$^{-/-}$ E13.5 MEFs. *, $P < 0.05$. *C,* succinylacetone (*SA*) treatment reduces CcO activity similarly (85% and 94%) in vector-control and ABCB6-overexpressing K562 cells. *Columns,* mean from three experiments; *bars,* SD. ***, $P < 0.001$.

peroxide, although ABCB6 provided only modest protection against a lower concentration of hydrogen peroxide. Taken together, these findings suggest that increased ABCB6 activity increases the availability of heme, thereby driving holo-catalase formation and enhancing the hydrogen peroxide tolerance of ABCB6-overexpressing cells.

Increased intracellular heme concentration has also been reported when the mitochondrial matrix-associated ABC transporter ABCB7 is overexpressed (14), although that study did not compare mitochondrial and cytosolic heme. We suspect that ABCB7 overexpression does not increase cytosolic heme or facilitate mitochondrial heme export, because the activity of catalase was unchanged by ABCB7 overexpression despite a >2-fold increase in cellular heme concentration (14). ABCB7 overexpression did increase the activity and quantity of the iron-sulfur cluster protein ferrochelatase (14), and *ABCB7* siRNA reduced the activity of aconitase, another such protein (26). This result is consistent with the view that ABCB7 facilitates iron-sulfur cluster formation (14, 26). We did not measure ferrochelatase, but we found that

another mitochondrial iron-sulfur cluster protein, succinate dehydrogenase, is unaffected by ABCB6 expression. Therefore, the effects of ABCB7 and ABCB6 on cellular enzymes differ markedly and seem to be closely linked to their transport activities.

Our findings link *de novo* heme synthesis and ABCB6 function to cytochrome *c* oxidase activity. Whereas inhibition of *de novo* heme synthesis reduces cytochrome *c* oxidase activity (13, 20, 22–24, 27), we showed that ABCB6 up-regulation of heme synthesis increases its activity. ABCB6 expression activated *de novo* porphyrin synthesis (23) and robustly elevated cytochrome *c* oxidase activity, whereas reduction of heme synthesis by either chemical means or *ABCB6* knockout reduced cytochrome *c* oxidase activity. We propose that, by increasing heme, ABCB6 either facilitates the assembly of cytochrome *c* oxidase or stabilizes it. Previous studies have shown that genetic defects in or chemical inhibition of heme synthesis affect the assembly of cytochrome *c* oxidase (13, 21–23, 25, 27). Heme A has been shown to facilitate cytochrome *c* oxidase assembly, and impaired cytochrome *c* oxidase assembly has been linked to reduced expression of its subunits under conditions of

heme deficiency (21–23, 27). We were unable to show elevated levels of heme A and do not know whether this result reflects methodologic problems or the absence of heme A elevation in the K562 cells. However, our results show that ABCB6 expression is likely to affect either the assembly of cytochrome *c* oxidase or the expression of some subunits, as these processes are affected by heme biosynthesis.

ABCB6 is overexpressed in cells exposed to chemical stressors (e.g., C2-ceramide), including cancer cells that have become resistant to cytotoxins. For example, the *ABCB6* gene is amplified in camptothecin-resistant cells (28, 29), and its overexpression is associated with multiagent resistance (29–31). We showed that enhanced ABCB6 expression alters the levels of cellular hemoproteins to provide a survival advantage. Although these cells were not generally resistant to cytotoxic challenge (sensitivity to etoposide was unaltered), they were resistant to cyanide and hydrogen peroxide stress. Furthermore, treatment with C2-ceramide, which enhances hydrogen peroxide concentration, up-regulates ABCB6 (32). Therefore, ABCB6 up-regulation may offer cells a survival advantage by enhancing catalase expression (33), and the effect of ABCB6 up-regulation on multiple hemoproteins may set the stage for acquisition of multiagent resistance.

In erythroid cells, heme export from the mitochondria requires ongoing protein synthesis (5, 6) and is facilitated by cytosolic proteins (7, 34). A number of these are heme-binding proteins, and some may be heme chaperones (4, 7, 35, 36). The absence of mitochondrial heme accumulation in ABCB6-overexpressing cells may reflect the coordinated action of a series of proteins within the mitochondria and cytosol that facilitate the transfer of heme from mitochondria to cytosol. This possibility is consistent with evidence that inhibition of protein synthesis causes mitochondrial heme accumulation (4). Importantly, however, not all heme-binding proteins facilitate heme efflux from the mitochondria. For example, glutathione transferases bind heme but do not mediate mitochondrial efflux (4). Thus, it is likely that, as heme synthesis increases (due to elevated PPIX levels), heme export leads to derepression of globin synthesis. Such a mechanism would be consistent with the increased hemoglobin observed in ABCB6-expressing cells. Moreover, given the increase in catalase activity, it would imply that mitochondrial efflux of heme does not limit the formation of holo-catalase.

Our results show that ABCB6 regulates not only *de novo* porphyrin biosynthesis (1) but also the concentration of cellular heme and hemoproteins in erythroid cells. This effect is directly illustrated by the increased concentration of hemoglobin, which depends on heme synthesis, the increased activity of cytochrome *c* oxidase (an intramitochondrial heme-dependent enzyme), and the increased activity of catalase (an extramitochondrial hemoprotein) in ABCB6-expressing cells. These findings were unexpected, because several factors might have limited the rate of formation of heme and hemoproteins: (*a*) ferrochelatase conversion of PPIX to heme (37, 38), (*b*) mitochondrial iron content, (*c*) energy-dependent transport of heme from the mitochondria (5, 39), and (*d*) the availability of cytosolic heme-binding proteins or chaperones. On the basis of our previous (1) and current findings, we propose a model that depicts how ABCB6 regulates heme concentration (Fig. 6). The mitochondrial conversion of PPIX to heme by ferrochelatase is probably a slow step (given the elevated concentration of PPIX), whereas the efflux of heme is very rapid. This model accounts for the mitochondrial accumulation of PPIX with no parallel change in mitochondrial heme and the role of ABCB6 as a key modulator of the terminal steps in heme synthesis. Whereas our studies have not excluded a small component of ABCB6 residing at the plasma membrane (as recently reported in ref. 16), it would be difficult to reconcile our findings of increased cytosolic heme with such a location. According to convention, a plasma membrane localized ABCB6 would be anticipated to efflux porphyrins, as was shown by Paterson and colleagues (16).
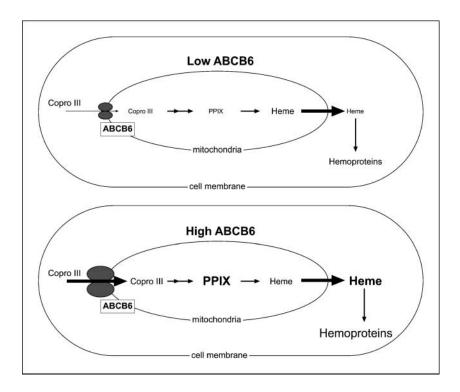

Figure 6. Model showing effect of the quantity of ABCB6 on heme and hemoproteins. The size of the lettering indicates relative concentration. The size of the arrows indicates relative rate of movement.

Our findings help to elucidate how ABCB6 activation produces fundamental changes within cells by altering their hemoprotein levels. They suggest that ABCB6 is frequently up-regulated in cells exposed to chemotherapeutic agents (29, 30) or environmental stress (32) as an adaptive response. This proposition is supported by findings that ABCB6 expression is correlated with response to multiple chemotherapeutic drugs (28). This dominant effect of ABCB6 has implications for mitochondrial diseases related to defects in cytochrome *c* oxidase and for other biochemical processes that depend on heme synthesis. These results may also provide insight into diseases associated with reduced ABCB6 expression (e.g., refractory thrombocytopenia; ref. 40).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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