Mitochondrial Translocator Protein (TSPO) Function Is Not Essential for Heme Biosynthesis*

Function of the mammalian translocator protein (TSPO; previously known as the peripheral benzodiazepine receptor) remains unclear because its presumed role in steroidogenesis and mitochondrial permeability transition established using pharmacological methods has been refuted in recent genetic studies. Protoporphyrin IX (PPIX) is considered a conserved endogenous ligand for TSPO. In bacteria, TSPO was identified to regulate tetrapyrrole metabolism and chemical catalysis of PPIX in the presence of light, and in vertebrates, TSPO function has been linked to porphyrin transport and heme biosynthesis. Positive correlation between high TSPO expression in cancer cells and susceptibility to photodynamic therapy based on their increased ability to convert the precursor 5-aminolevulinic acid (ALA) to PPIX appeared to reinforce this mechanism. In this study, we used TSPO knock-out (Tspo−/−) mice, primary cells, and different tumor cell lines to examine the role of TSPO in erythropoiesis, heme levels, PPIX biosynthesis, phototoxic cell death, and mitochondrial bioenergetic homeostasis. In contrast to expectations, our results demonstrate that TSPO deficiency does not adversely affect erythropoiesis, heme biosynthesis, biocconversion of ALA to PPIX, and porphyrin-mediated phototoxic cell death. TSPO expression levels in cancer cells do not correlate with their ability to convert ALA to PPIX. In fibroblasts, we observed that TSPO deficiency decreased the oxygen consumption rate and mitochondrial membrane potential (∆Ψm) indicative of a cellular metabolic shift, without a negative impact on porphyrin biosynthetic capability. Based on these findings, we conclude that mammalian TSPO does not have a critical physiological function related to PPIX and heme biosynthesis.

Mammalian translocator protein (TSPO), previously known as the peripheral benzodiazepine receptor (1), is a highly conserved protein enriched in the outer mitochondrial membrane (2). Despite extensive efforts to characterize TSPO, its precise physiological function remains elusive (3, 4). High levels of TSPO expression in steroidogenic cells, its localization to the outer mitochondrial membrane, and increased steroid production upon pharmacological binding led to the primary prospective model that TSPO was a mitochondrial cholesterol transporter essential for steroidogenesis (5). In recent studies using precise genetic tools, we and others have systematically refuted the involvement of TSPO in this process (6–10). Similarly, copurification of TSPO with putative members of the mitochondrial permeability transition pore (MPTP) (11) and effects mediated by TSPO binding drugs on modulating apoptosis (12, 13) resulted in a secondary model that TSPO was associated with MPTP function and cell death (14). Again, recent discovery of the molecular identity of MPTP (15) and direct testing of MPTP function in the absence of TSPO (16) disputed its direct involvement in cell death processes (reviewed in Ref. 4). These new developments have shifted focus of TSPO function to yet another molecule considered to be an endogenous ligand, protoporphyrin IX (PPIX) (17).

Binding of porphyrins to TSPO has been a consistent property reported in bacteria (18), plants (19), and animals (17). In Rhodobacter sphaeroides, TSPO was found localized to the outer membrane (18) and played a role in negatively regulating photosynthesis genes in response to oxygen (20). Examining tetrapyrrole metabolism after providing the precursor ALA to different Tspo mutants of R. sphaeroides led to a conclusion that TSPO facilitated the export of excessive intermediates in the tetrapyrrole pathway (21). In Bacillus cereus, TSPO mediated a light-induced degradation of PPIX (22). In Arabidopsis thaliana, it was observed that TSPO attenuated ALA-induced porphyria through a potential scavenging mechanism (23). In Danio rerio embryos, pharmacological binding of TSPO protein using PK11195 (N-butyl-2-yl-1-(2-chlorophenyl)-N-methylisouquinoline-3-carboxamide) or morpholino antisense oligonucleotides resulted in a loss of circulating erythrocytes

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2 The abbreviations used are: TSPO, translocator protein; PPIX, protoporphyrin IX; PDT, photodynamic therapy; ALA, 5-aminolevulinic acid; MPTP, mitochondrial permeability transition pore; OCR, oxygen consumption rate; PK11195, N-butyl-2-yl-1-(2-chlorophenyl)-N-methylisouquinoline-3-carboxamide; Alas, ALA synthase; Adal, ALA dehydratase; Hmbh, hydroxymethylbilane synthase; Uros, uroporphyrinogen III synthase; Urod, uroporphyrinogen decarboxylase; Cpox, coproporphyrinogen oxidase; Ppox, protoporphyrinogen oxidase; Fech, ferrochelatase; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; TMRM, tetramethylrhodamine methyl ester.
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(24). In Gallus gallus, pharmacological binding of Tspo using PK1195 decreased globin levels indicating a function in the regulation of heme availability for the assembly of functional hemoglobin (25).

Early studies using Tspo-binding drugs have suggested a functional link between mammalian (Mus musculus and Homo sapiens) Tspo and the induction of hemoglobin synthesis (26, 27). Subsequent studies identified that Tspo could bind the heme precursor PPIX with nanomolar affinity (17) and that Tspo induction that was concomitant with heme biosynthetic enzymes, suggesting a possible involvement in porphyrin transport important for heme biosynthesis (28, 29). It was also demonstrated that knockdown of Tspo expression resulted in mitochondrial accumulation of exogenous PPIX, suggesting that Tspo could be involved in heme metabolism (30).

Pathological overexpression of Tspo has been reported in several cancers like colon, breast, and skin cancers (31, 32). A strong correlation between the aggressive cancer phenotype and expression levels of Tspo has been established in several studies (33–35). Cancer cells also show an increased ability to produce endogenous PPIX when supplemented with ALA (36, 37), a photosensitization mechanism that forms the basis of porphyrin-mediated PDT. In this context, experiments have suggested that Tspo plays a major role in PDT by its action on porphyrins (38, 39).

In search of a functional basis for this proposed relationship between mammalian Tspo and PPIX, we use genetic models, Tspo gene deleted (Tspo−/−) mice, Tspo−/− primary cells, and Tspo−/− cancer cells to investigate Tspo-specific actions. Our results demonstrate that Tspo is not essential for porphyrin and heme biosynthesis or porphyrin-mediated phototoxic cell death, but that Tspo deficiency affects mitochondrial energy homeostasis that may impact multiple cellular events and responses.

Experimental Procedures

Tspo−/− Mice—Generation and validation of Tspo−/− mice has been previously described (7). Animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Institutional Animal Care and Use Committee of Cornell University approved all procedures.

Cells—All cell culture procedures were performed at 37 °C under 5% CO2. Primary murine fibroblasts were cultured from day 14 embryos in growth medium (DMEM containing 10% FBS and 1% nonessential amino acids). In brief, embryos were aseptically collected, and carcasses were minced and plated in cell culture dishes for attachment and growth. The cells were passaged once and frozen for use in experiments. Generation and culture of Tspo deleted MA-10 cells (MA-10:TspoΔ/Δ cells) has been previously described (8). Human colon cancer cell lines were obtained from ATCC and cultured using recommended methods. Cell lines HCT116 and HT29 were cultured in McCoy’s 5A medium containing 10% FBS. Cell line LOVO was cultured in F12K medium containing 10% FBS. Cell line DLD-1 was cultured in RPMI1640 containing 10% FBS.

Hematology and Bone Marrow Histology—Blood was collected in heparinized tubes (BD Biosciences). Erythrocyte counts, platelet, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were obtained using an automated analyzer (Advia 120, Siemens). Erythrocyte counts were also confirmed manually using a hemocytometer. Femurs were processed for histology by fixation in 10% formaldehyde, followed by slow decalcification and paraffin embedding. Thin 4–μm sections were cut and stained using hematoxylin and eosin. Bone marrow cytology was assessed on brush preparations made from the marrow and stained using modified Wright’s stain. Marrow hematopoietic cellularity and percentage of erythroid cells were assessed on bone marrow histology and cytology preparations by a board certified veterinary pathologist, and the percentage of erythroid precursors was calculated. Images were captured under a light microscope (DM1000; Leica) using a color camera (ICC 50HD; Leica).

Measurement of Heme in Tissues—Heme content was determined using a fluorometric method as described (40). Liver, spleen, and bone marrow samples were homogenized in 1% Triton X-100 in Tris-buffered saline. Homogenized samples were centrifuged at 5000 × g for 10 min. 1 μl of the homogenate was added to 500 μl of saturated (2 m) oxalic acid and boiled for 30 min to produce PPIX from heme. Samples were then centrifuged at 10,000 × g for 10 min at 4 °C, and PPIX fluorescence in the supernatant was measured using a fluorescence spectrofluorometer (Infinite 200; Tecan), under excitation at 400 nm and emission at 660 nm, and concentrations were calculated using a standard curve. Pre-existing non-heme PPIX was subtracted from total heme by using a duplicate, unboiled sample. Heme values were normalized to protein content in each sample.

PPIX Uptake and Phototoxicity—For estimating PPIX uptake, fibroblasts treated with 0 (control), 0.5, 1, or 1.5 μM PPIX (Sigma) for 4 h in serum-free medium were collected by trypsinization. The trypsin was neutralized using 0.7 mg/ml type II-O trypsin inhibitor (Sigma) in DMEM to avoid exposure to serum. Cells were then resuspended, fixed using 1% formaldehyde, and assayed using a flow cytometer at an excitation of 488 nm and an emission range of 620–630 nm (Gallios; Beckman Coulter) to estimate the median PPIX uptake by individual cells. For evaluating phototoxicity, fibroblasts were treated with PPIX (0 (control), 0.5, 1, or 1.5 μM) for 4 h as described above were exposed to light at 450 (± 60)-nm wavelength at 160, 240, or 320 mW using an 800 milliwatt mercury lamp light path fitted with a band-pass filter and a neutral density filter (OD 1.0). After exposure, fibroblasts were provided with growth medium and incubated for 6 h before labeling using propidium iodide (20 μg/ml) and Hoescht 33342 (1 μg/ml) to determine live and dead cells. Populations were counted after acquiring images with an inverted epifluorescence microscope (DM3000; Leica) using a monochromatic cooled camera (DFC365FX; Leica).

Bioconversion of ALA to PPIX—For in vivo experiments, ALA (25 mg/ml in PBS; Sigma) was administered to mice (250 mg/kg body weight intraperitoneal) and euthanized at 0 (baseline), 1, 4, or 8 h after ALA administration. Plasma, bone marrow (femurs), livers, and spleens were collected for estimating PPIX concentrations. For estimation of PPIX, samples were extracted using 1:1 methanol-1N perchloric acid (MeOH-PCA) on ice for
10 min, and the lysates were cleared by centrifugation at 10,000 \times g for 10 min at 4 °C. Supernatants (100 \mu l) for each sample were analyzed for PPIX in black 96-well plates using a fluorescence spectrophotometer as described for heme, and concentrations were calculated using a standard curve. For plasma, data were represented as PPIX concentration per milliliter. For tissues, acidic pellets were neutralized with 1.5 M Tris acetate buffer and used to measure protein content using the bicinchoninic acid assay; PPIX concentrations were normalized to protein content. In a separate experiment, at 1 h after ALA administration, bone marrow (femurs) were collected for RNA extraction and quantitative PCR as described below.

For in vitro experiments, fibroblasts (density of 2,500 cells/cm²), MA-10 cells (5,000 cells/cm²), and colon cancer cell lines (5,000 cells/cm²) were treated with 1 mM of ALA and incubated for baseline and 24 h. After incubation, cells were collected by trypsination followed by neutralization using trypsin inhibitor in DMEM. Cells were lysed in 1:1 MeOH-PCA, and PPIX fluorescence was estimated as described above for tissue samples.

Western Blots—Samples were processed in Laemmli sample buffer as previously described (41), and protein concentrations were determined using a bichionic acid assay. Equal amounts of protein (25–50 \mu g/sample) were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were then blocked using 5% nonfat dry milk in Tris-buffered saline containing 0.2% Tween 20. Incubations were carried out using a rabbit anti-TSPO monoclonal antibody (Abcam), a rabbit anti-IDH2 monoclonal antibody (Abcam), or a mouse anti-VDAC1 monoclonal antibody (Abcam) and a control mouse anti-actin monoclonal antibody (Li-Cor). Fluorescent IRDye800 goat anti-rabbit IgG and IRDye680 goat anti-mouse IgG were used for fluorescent labeling; membranes were imaged using a laser fluorescence scanner (Odyssey; Li-Cor).

PPIX and Mitochondrial Colocalization—Fibroblasts cultured in glass-bottomed 35-mm dishes (MatTek corporation) were treated with 1 \mu M PPIX in serum-free medium for 4 h. MitoTracker Green (Life Technologies) was added to label the mitochondria in live cells for the final 30 min of this incubation. Labeled cells were then provided with fresh medium, and high resolution images were acquired using a confocal microscope (Meta 510; Zeiss). Image analyses for colocalization of PPIX and MitoTracker were performed using the intensity correlation plugin in ImageJ (National Institutes of Health) (42) and calculating the Mander’s overlap coefficient and Pearson’s coefficient. At least 40 cells for each genotype were imaged and analyzed.

Quantitative PCR—Gene expression analyses using quantitative PCR were performed for both in vitro (fibroblasts and MA-10 cells) and in vivo (bone marrow) samples. Total RNA was extracted using TRIzol reagent (Life Technologies); reverse transcription of 1.5 \mu g of total RNA was performed using Multiscribe reverse transcriptase (Life Technologies). Gene expression assays for the different samples were performed using SYBR-green using intron-spanning primers for ALA synthase (Alas: 5'-TGTCCGGCTGTGACGAATG-3' and 5'-GGC-ATCATCTAGCAGGTG-3'), ALA dehydratase (Alad: 5'-GTTGTGACAGCGGGTACCT-3' and 5'-AGCTGGTTTA-CGCCATACTCTG-3'), hydroxymethylbilane synthase (Hmbs: 5'-ATGAGGGTGATCTGAGTG-3' and 5'-TTCTTCC- CGTGGTGACATA-3'), uroporphyrinogen III synthase (Uros: 5'-CCAGCATCGAGTTTCCATGC-3' and 5'-GTA-CTGGGGCAATGCTG-3'), uroporphyrinogen decarboxylase (Urod: 5'-GCGTGGACCTTCAATTCC-3' and 5'-CCTGTCTCAGCAGTAAAGC-3'), coproporphyrinogen oxidase (Cpox: 5'-GCATTATCTGCTATGGG-3' and 5'-AGTTCTTTAGGATCGCTGG-3'), proporphyrinogen oxidase (Ppox: 5'-ATGGGCGGACTGTGATAGTA-3' and 5'-CTTGCTGCCCTCACTAAGA-3'), ferrochelatase (Fech: 5'-CAGACAGCTAGGCTCTAAGG-3' and 5'-CACAGCCTGTGGACTGATG-3'), and Tspo (5'-TGCCGTTCACTCTGCA-3' and 5'-AATCCACGATGCTGAATCCT-3'). Samples were analyzed after normalization to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH: 5'-GCCCTCGGTGTTCTTACC-3' and 5'-GCCGCT- TTCACACACCTTC-3') or TATA box binding protein (Tbp: 5'-CCTGTGACCCCTCACTGAGC-3' and 5'-AACAGCC- AAGATTACGCGTAGA-3'). Relative quantifications of fold change were performed comparing \( \Delta \Delta C_t \) values from individual samples by applying the 2 \(-\Delta \Delta C_t \) method (43). Because of difficulty in designing intron-spanning primers, translocator protein 2 (Tspo2) expression was quantified using a TaqMan probe (Mm01281420_m1) with normalization to Gapdh (4352339E) (Life Technologies).

Adenoviral Expression of Tspo2—Adenoviruses that express Tspo2 were generated using the AdEasy system (44). In brief, Tspo2 cDNA was cloned from a bone marrow sample into a shuttle vector (pAd-CMV), linearized using PacI, and transformed into AdEasier bacteria containing the adenoviral backbone plasmid (pAdEasy-1). Adenoviruses were packaged by transfection into HEK-293A cells and amplified for two cycles in the same cell line. Titers were estimated by quantifying viral DNA, and infection rates were assessed by Tspo2 mRNA expression in the target cells by quantitative PCR. Control adenoviruses expressing tdTomato (Vector Biolabs) were amplified in HEK-293A cells and used as matched titers. Fibroblasts infected with adenoviral Tspo2 or tdTomato were used after 48 h for experiments evaluating the bioconversion of ALA to PPIX as described above.

Oxygen Consumption Rate—For measurements of mitochondrial respiration, oxygen consumption rates (OCR) were measured using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience). Fibroblasts (Tspo2/+/ and Tspo2-/-) were plated at a density of 30,000 cells in the XF24 cell culture microplates in regular culture medium (10% FBS, 25 \mu M glucose, 1 \mu M pyruvate) overnight. One hour before assay, cells were washed and incubated in XF assay medium (no bicarbonate) supplemented with 25 \mu M glucose and 1 \mu M pyruvate at 37 °C in a CO2-free atmosphere. OCR was measured real time every 3–5 min. Oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), antimycin A, and rotenone were sequentially injected into each well to assess basal respiration, coupling of respiratory chain, proton leak, and mitochondrial spare respiratory capacity.
Mitochondrial Volume, Mass, and Membrane Potential—Fibroblasts, Tspo^{fl/fl} and Tspo^{−/−}, were seeded on 35-mm glass-bottomed dishes (MatTek) overnight in high glucose DMEM, 10% FBS. Cells were stained with 100 nM MitoTracker Green FM at 37 °C for 1 h in the dark, washed with PBS, and changed to phenol red-free high glucose DMEM, 10% FBS, 10 mM HEPES for live cell imaging using Zeiss LSM 510 confocal microscopy. Images were collected as z-stacks and analyzed for volume after adjustment of threshold using three-dimensional Manager plugin in ImageJ (45).

For mitochondrial membrane potential measurement, cells were costained with 100 nM MitoTracker Green FM and 20 nM tetramethylrhodamine methyl ester (TMRM) and imaged as intensity mean; RDW, red blood cell distribution width; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, cell hemoglobin concentration; CHCM, cell hemoglobin concentration; for comparisons of Δψm between the two genotypes. Mitochondrial masses were also confirmed by estimating the amounts of mitochondrial proteins IDH2 and VDAC1 using Western blots (as described above).

**PK11195 Treatments**—For ALA to PPIX bioconversion, fibroblasts were treated with PK11195 (1 μM) in 0.1% DMSO (control) in medium containing 1 mM of ALA and incubated for 24 h. After incubation, cells were collected and lysed, and PPIX fluorescence was estimated as described above. For measuring Δψm, fibroblasts treated with PK11195 (0 and 100 nM and 1 μM) were incubated for 60 min and stained with MitoTracker Green FM and TMRM, and intensities were measured using flow cytometry as described above.

**Results**—Bone marrow histology and evaluation of erythroid cells. A, sections of Tspo^{fl/fl} and Tspo^{−/−} mice femur bone showing the marrow. Histological evaluation of the marrow showed no difference in overall hematopoietic cellularity. Scale bar, 200 μm. Inset, bone marrow cytology; there was no difference in relative density of erythroid precursor cells. Scale bar, 15 μm. B, enumeration of erythroid cells showed no differences in the percentage of erythroid precursors between the two groups (n = 3/group).

**TABLE 1**

| Parameters                  | Tspo^{fl/fl} | Tspo^{−/−} | P value |
|-----------------------------|--------------|------------|---------|
| Erythrocytes (10^{12} cells/μL) | 9.8 ± 0.05   | 9.8 ± 0.15 | 1.0000  |
| Platelets (10^{11}/μL)      | 1284.4 ± 182.24 | 1508.6 ± 196.58 | 0.4272  |
| Hemoglobin (g/dL)           | 15.7 ± 0.15   | 15.8 ± 0.16 | 0.5028  |
| Hematocrit (%)              | 52.8 ± 0.66   | 52.0 ± 0.44 | 0.3466  |
| MCV (fL)                    | 54.20 ± 0.8   | 53.00 ± 1.09 | 0.4021  |
| MCH (pg)                    | 15.6 ± 0.24   | 16.0 ± 0.31 | 0.3466  |
| MCHC (g/dL)                 | 29.0 ± 0.00   | 30.0 ± 0.31 | 0.3411  |
| CHCM (g/dL)                 | 27.0 ± 0.00   | 27.4 ± 0.24 | 0.1411  |
| RDW (%)                     | 12.6 ± 0.24   | 12.4 ± 0.25 | 0.5796  |
levels were not different between $Tspo^{β/β}$ and $Tspo^{−/−}$ livers and spleens (Fig. 3, B and C). However, heme levels were modestly higher but significant in bone marrow from $Tspo^{−/−}$ mice compared with $Tspo^{β/β}$ cohorts (Fig. 3D).

Systemic Conversion of ALA to PPIX in $Tspo^{−/−}$ Mice—Baseline PPIX concentrations in plasma, and tissue lysates (bone marrow, livers and spleens) were not different between $Tspo^{β/β}$ and $Tspo^{−/−}$ mice (Fig. 4). After ALA administration, increases in PPIX levels at 1, 4, and 8 h were similar in both $Tspo^{β/β}$ and $Tspo^{−/−}$ mice in plasma and all tissues.

**TSPO Deficiency Does Not Affect PPIX Uptake and Localization**—When $Tspo^{β/β}$ and $Tspo^{−/−}$ fibroblasts were treated with PPIX, the extent of PPIX localization to the mitochondria computed as overlap with MitoTracker® Green fluorescence was identical between the two genotypes (Fig. 5, A–C). Treatments of $Tspo^{β/β}$ and $Tspo^{−/−}$ fibroblasts with increasing concentrations of PPIX showed dose-dependent increases in intracellular fluorescence that were not different between the two genotypes (Fig. 5D). PPIX uptake by fibroblasts approached saturation at 1 μM PPIX treatment, with no difference observed when treatment was increased to 1.5 μM PPIX (Fig. 5D).

**TSPO Deficiency Does Not Affect Porphyrin-mediated Phototoxicity**—Without PPIX treatment, exposure of both $Tspo^{β/β}$ and $Tspo^{−/−}$ fibroblasts to light at 450 ± 60 nm wavelengths with increasing energies (160, 240, and 320 mJ) did not induce phototoxicity and cell death (Fig. 6). When these $Tspo^{β/β}$ and $Tspo^{−/−}$ fibroblasts were treated with PPIX, they displayed different levels of cell death that increased with both increasing concentration of PPIX and increasing energy of light (up to 95% cell death was observed with 1.5 μM of PPIX treatment and 320 mJ light energy). However, no significant differences in PPIX-mediated phototoxic cell death were observed between $Tspo^{β/β}$ and $Tspo^{−/−}$ fibroblasts (Fig. 6).

**Bioconversion of ALA to PPIX Is Not Changed in $Tspo^{−/−}$ Fibroblasts**—Baseline PPIX concentration in $Tspo^{β/β}$ and $Tspo^{−/−}$ fibroblast lysates were not different (Fig. 7A). After ALA treatment, PPIX concentrations in fibroblast lysates showed a significant increase with no statistical difference between the two genotypes (Fig. 7A). Expression of genes involved in PPIX biosynthesis indicated some differences between $Tspo^{β/β}$ and $Tspo^{−/−}$ fibroblasts (Fig. 7B). Baseline expression of Alas, Hmbs, Urod, Cpox, Ppox, and Fech were significantly lower in $Tspo^{−/−}$ compared with $Tspo^{β/β}$ fibroblasts; baseline expression of Alad and Uros was not different between the two genotypes. After ALA treatment, expression of Alas, Alad, Hmbs, and Cpox was not different between the two genotypes; expression of Uros, Urod, Ppox, and Fech was significantly lower in $Tspo^{−/−}$ compared with $Tspo^{β/β}$ fibroblasts. Expression of Tspo did not show any changes with ALA treatment in $Tspo^{β/β}$ fibroblasts. Tspo2 expression was not detected in $Tspo^{β/β}$ and $Tspo^{−/−}$ fibroblasts at both baseline and after treatment with ALA (not shown).

**Bioconversion of ALA to PPIX Is Not Changed in MA-10: $Tspo^{λ/λ}$ Cells**—Baseline PPIX concentration in MA-10 cell lysates showed a modest but significantly lower level in MA-10: $Tspo^{λ/λ}$ cells compared with MA-10 cells (Fig. 8A). After ALA treatment, PPIX concentrations in cell lysates showed a significant increase with no difference between MA-10:$Tspo^{λ/λ}$ cells

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**FIGURE 2.** Expression of genes involved in PPIX synthesis in $Tspo^{−/−}$ bone marrow. A, schematic showing the different enzymatic steps involved in the conversion of ALA to PPIX and heme. The enzymes were ALA synthase (Alas), ALA dehydratase (Alad), hydroxymethyl bilane synthase (Hmbs), uroporphyrinogen III synthase (Uros), uroporphyrinogen decarboxylase (Urod), coproporphyrinogen oxidase (Cpox), protoporphyrinogen oxidase (Ppox), and ferrochelatase (Fech). The intermediates were porphobilinogen (PBG), hydroxymethyl bilane (HMB), uroporphyrinogen III (UROG III), coproporphyrinogen III (CPG III), and protoporphyrinogen IX (PPGIX). B, expression levels of transcripts coding enzymes involved in the conversion of ALA to PPIX in $Tspo^{β/β}$ and $Tspo^{−/−}$ bone marrow at both baseline and after treatment with ALA. A, b, and c indicate $p < 0.05$ ($n = 6$/group).
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FIGURE 3. Heme levels in tissues from Tspo−/− mice. A, PPIX standard and a representative analytical spleen sample showing the emission spectrum of PPIX with excitation at 400 nm; two emission maxima at 605 and 660 nm specific for PPIX are observed in both standard and prepared sample. B–D, normalized baseline heme levels in tissues (liver, spleen, and bone marrow) from Tspo+/+ and Tspo−/− mice. *, p < 0.05 (n = 6/group).

FIGURE 4. PPIX levels in plasma and tissues from Tspo−/− mice. Panels show normalized PPIX fluorescence in blood plasma (A), liver (B), spleen (C), and bone marrow (D), at baseline and at 1, 4, and 8 h after administration of ALA (n = 6–8/group).

and MA-10 cells (Fig. 8A). Expression of genes involved in PPIX biosynthesis indicated some differences between MA-10: Tspo−Δ/Δ cells and MA-10 cells (Fig. 8B). Baseline expression of Alad, Uros, and Urod were significantly higher in MA-10: Tspo−Δ/Δ cells compared with MA-10 cells, and these differences did not change after ALA treatment. Baseline expression of Alas was not different between MA-10:Tspo−Δ/Δ cells and MA-10 cells, but expression significantly decreased upon ALA treatment in both genotypes. Baseline expression of Hmbs, Cpxo, and Ppox was not different between MA-10:Tspo−Δ/Δ cells and MA-10 cells, but a significant increase in expression was observed after ALA treatment only in MA-10:Tspo−Δ/Δ cells compared with MA-10 cells. Baseline Fecnh expression was significantly decreased in MA-10:Tspo−Δ/Δ cells compared with MA-10 cells; this difference was also seen after ALA treatment. Expression of Tspo showed a modest but significant decrease with ALA treatment in MA-10 cells. Tspo2 expression was not observed in MA-10 cells and MA-10:Tspo−Δ/Δ cells at both baseline and after treatment with ALA (not shown).

TSPO Expression Does Not Correlate to PPIX Production by Colon Cancer Cells—In comparing fibroblasts and MA-10 cells with different human colon cancer cell lines, TSPO expression was highest in MA-10 cells and weakest in fibroblasts; among the colon cancer cell lines, DLD1 > HT29 > HCT116 ≥ LOVO (Fig. 9A). Expression of IDH2, used as an indirect measure of mitochondrial mass in the different colon cancer lines, showed a range of differences between the cell lines, suggesting varied mitochondrial masses; relative expression of ACTB was also not perfectly consistent for baseline normalization between the cell lines (Fig. 9A). PPIX synthesis after ALA treatment in the colon cancer cell lines showed levels decreasing in order from HT29 > HCT116 > DLD1 > LOVO cells (Fig. 9B). In LOVO cells, we did not observe enhanced PPIX production after ALA treatment. These observations support the results seen in MA-10 cells, suggesting that the efficacy of PPIX synthesis after ALA treatment is not related to TSPO expression in colon cancer cells.

Exogenous Tspo2 Expression Does Not Change ALA to PPIX Bioconversion in Fibroblasts—There was no expression of Tspo2 observed in fibroblasts and MA-10 cells (Fig. 10A). Exogenous adenoviral Tspo2 expression in Tspo−/− fibroblasts resulted in ~8-fold higher expression levels compared with endogenous expression observed in the bone marrow. PPIX synthesis before and after ALA treatment was not different between Tspo−/− and Tspo−−/− fibroblasts after exogenous expression of Tspo2 (Fig. 10B). In control tdTomato expression, ALA treatment showed a modest but significant increase in PPIX levels in Tspo−/− fibroblasts. However, this increase was not significantly different compared with Tspo2 expressing Tspo−/− fibroblasts. These results suggest that Tspo2 expression does not have a positive or negative effect on PPIX production from ALA.

Mitochondrial Homeostasis Is Altered in Tspo−/− Fibroblasts—Comparing primary fibroblasts from Tspo−/− and Tspo−−/− mice showed that baseline and maximal OCR were significantly lower with the loss of TSPO (Fig. 11A). However, the percentages of oxidative phosphorylation coupling efficiencies were unchanged in Tspo−/− compared with Tspo−−/− fibroblasts (Fig. 11B), but absolute values were significantly lower in Tspo−/− fibroblasts (not shown). There was a decrease in calculated ATP production in Tspo−/− compared with Tspo−−/− fibroblasts (Fig. 11C). Proton leak was also decreased in Tspo−/− compared with Tspo−−/− fibroblasts (Fig. 11D). The spare respiratory capacity was significantly reduced in Tspo−/− compared with Tspo−−/− fibroblasts (Fig. 11E). These findings suggest a significant shift in mitochondrial homeostasis in Tspo−/− fibroblasts that could affect multiple mitochondrial and cellular functions.
Mitochondrial Membrane Potential Is Decreased in Tspo⁻/⁻ Fibroblasts—Assessment of ΔΨm showed that Tspo⁻/⁻ fibroblasts had significantly lower baseline values compared with Tspo⁺/⁺ fibroblasts, and this difference was increased after inhibition of ATP synthase by oligomycin and completely dissipated as expected after FCCP in both genotypes (Fig. 12, A and B). Given the scale of these changes as a result of TSPO deletion, we verified that mitochondrial volume and mass were not different between Tspo⁻/⁻ and Tspo⁺/⁺ fibroblasts (Fig. 12, C–E). Differences observed in Tspo⁻/⁻ fibroblasts corroborate that loss of TSPO impacts mitochondrial homeostasis in primary cells.

PK11195 Does Not Affect ALA to PPIX Bioconversion but Has Off Target Effects Affecting ΔΨm—Treatment with PK11195 [1 μM, the concentration used in the zebrafish study (24)], did not affect PPIX production after ALA treatment in both Tspo⁺/⁺ and Tspo⁻/⁻ fibroblasts (Fig. 13A). However, this same concentration of PK11195 significantly decreased ΔΨm in both Tspo⁺/⁺ and Tspo⁻/⁻ fibroblasts (Fig. 13B), indicating that PK11195 treatment can result in off target effects at this concentration. Baseline values for ΔΨm measured using flow cytometry also showed a significantly lower ΔΨm in Tspo⁻/⁻ fibroblasts compared with Tspo⁺/⁺ fibroblasts (Fig. 13B), consistent with the microscopic quantitation (Fig. 12A and B).

Discussion

Based on its conserved PPIX binding property (17), it was proposed that the ancestral role for TSPO could be enzyme catalysis of porphyrins, albeit only in the presence of light (46). Purified TSPO protein from Chlorobium tepidum (CtTSPO)
and *B. cereus* (*BcTSPO*) indicated a property of photo-oxidative PPIX degradation (22, 46). In contrast, chordate TSPO (fish, birds, and mammals) has been suggested to be important for PPIX transport and heme biosynthesis (24, 25, 28, 47). The PPIX binding property of TSPO was also considered the basis of ALA-based PDT for cancers (38, 39, 48). Diverging from these conclusions, results in this manuscript suggest that TSPO does not have a physiological effect on PPIX metabolism and may not be involved in PDT.

Deficiency of TSPO did not affect heme synthesis and erythropoiesis in mice, suggesting an evolutionary shift distinct from its hematopoietic function reported in zebrafish (49). Bone marrow, the primary site of erythropoiesis, also expresses the TSPO paralog TSPO2 that can be found only in birds and mammals (49). TSPO2 has 35% homology to TSPO and is almost exclusively localized to the bone marrow (49). However, TSPO2 localization to the endoplasmic reticulum (49) suggests that it may not be involved in a mitochondrial function that is redundant to TSPO. Fibroblasts and MA-10 cells do not express TSPO2. Moreover, we confirmed that TSPO2 does not affect PPIX synthesis using adenovirus-induced Tspo2 expression in fibroblasts, ruling out the possibility of functional redundancy. In support, expression of the heme biosynthetic gene *Fech* showed a compensatory baseline increase in expression in *Tspo*<sup>−/−</sup> bone marrow, suggesting that TSPO deletion...
did induce a shift in mitochondrial homeostasis. However, this shift in homeostasis toward increased heme synthesis did not alter physiological hemoglobin concentration measured in circulation. Moreover, when \( Tspo^{-/-} \) mice were treated with ALA, there were no differences in maximal PPIX production in plasma, liver, spleen, and bone marrow. Although \( Tspo^{-/-} \) bone marrow (functionally akin to previous reports of \( Tspop^{fl/fl} \) mice) had no differences in PPIX production, it is possible that administration of ALA promotes erythroid differentiation in the bone marrow, as observed in the widely used in vitro K562 erythroid differentiation model (50–52). A \( Tspo \) up-regulation response as reported in erythroid cells was not observed in fibroblasts or MA-10 cells, also suggesting that this is specific for the bone marrow.

To examine PPIX uptake and porphyrin-mediated phototoxicity in a system that expresses TSPO (but not TSPO2), we used primary fibroblasts as a model (53) to examine mechanisms. We found that TSPO deficiency did not affect the extent of PPIX association with the mitochondria and the level of uptake by fibroblasts. Moreover, PPIX-mediated phototoxic cell death was not different between \( Tspop^{fl/fl} \) and \( Tspo^{-/-} \) fibroblasts. Although these observations were rather unexpected given that previous studies reported the contrary in other model systems (12, 30, 54), they corroborated a recent report demonstrating that PPIX accumulation was not different in between \( Tspop^{fl/fl} \) and \( Tspo^{-/-} \) mitochondria isolated from the liver (16).

Early studies have established that CPOX is present in the mitochondrial intermembrane space and that coproporphyrinogen III needs to cross the outer mitochondrial membrane to gain access to this enzyme (55). Although weaker than its affinity for PPIX, TSPO was demonstrated to bind coproporphyrinogen III (28). Because TSPO-binding chemicals appeared to block the conversion of coproporphyrinogen III to PPIX.
gen III to PPIX via intermediate protoporphyrinogen IX, a possible function for TSPO as a porphyrin transporter was proposed (29). In support, it was demonstrated that TSPO was directly involved in PPIX transport in an experiment using bacterial protoplasts expressing murine TSPO (47). In the present study, when we examined the ability of fibroblasts to synthesize PPIX from precursor ALA, we found no difference between 
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\text{Tspo}^{+/+}/H11002\text{/}
\]
and 
\[
\text{Tspo}^{-/-}\text{/}
\]
cells. The exact same experiment in MA-10 cells (that also do not express TSPO but express 14-fold higher TSPO compared with fibroblasts) showed no difference between MA-10 and MA-10: 
\[
\text{Tspo}^{+/+}/H11002\text{/}
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cells. These results agreed with our in vivo observations in 
\[
\text{Tspo}^{-/-}\text{/}
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mice and confirmed that TSPO is not involved in PPIX biosynthesis.

Provided the prognostic significance proposed for TSPO expression in colon cancer (56) and evidence that TSPO density in cancers parallels their susceptibility to porphyrin-mediated phototoxicity (54), we examined TSPO and PPIX synthesis in cancer cells. Comparison of different human colon cancer cell lines showed that TSPO expression had no correlation with PPIX synthesis capability, similar to observations in fibroblasts and MA-10 cells. Therefore, earlier reports indicating that TSPO directs mitochondrial porphyrin transport as part of the biosynthetic machinery (29, 47) are not substantiated. Recent
identification of an essential porphyrin transporter ABCB6 (ATP-binding cassette, subfamily A (ABC1), member 6) at the outer mitochondrial membrane could explain the mechanism of mitochondrial porphyrin uptake and heme biosynthesis (57), without a role for TSPO in this process.

Although the PPIX binding property of TSPO is highly conserved and photo-oxidative degradation of PPIX could be considered an enzymatic function for TSPO, the physiological relevance of this slow and light-dependent activity in multicellular organisms appears unclear (58). Recent high resolution structural studies on MmTSPO (M. musculus) (59), RsTSPO (R. sphaeroides) (60), and BcTSPO (22) highlight limited similarities between vertebrate (MmTSPO) and the bacterial forms (RsTSPO and BcTSPO) with respect to PPIX binding residues, side chain interactions, and dimerization (58). In addition to the <30% identity, conserved residues that are organized on the outside of MmTSPO are reversed in BcTSPO and RsTSPO. Moreover, MmTSPO was found to be a monomer, RsTSPO appears to form an obligate dimer, and BcTSPO could also dimerize but does so with a distinct interface, suggesting evolutionary separations if this organization is indeed functionally important. Although the PPIX photo-oxidative degradation mediated by CtTSPO and BcTSPO are often discussed in broad terms, there is no functional evidence for this property in MmTSPO. Experiments that used the vertebrate XtTSPO (Xenopus tropicalis), and HsTSPO (H. sapiens) A147T variant showed that the catalytic photo-oxidative degradation of PPIX is conserved in XtTSPO but not in HsTSPO (22). The photo-oxidative degradation property of the wild type HsTSPO and MmTSPO remains to be tested. Moreover, light-mediated reactions would be quite unusual for most tissues that express MmTSPO or HsTSPO. Therefore, results obtained for bacterial TSPO need to be extrapolated with caution, because they may not be conserved or relevant in higher vertebrates.

We find that TSPO deficiency significantly decreases OCR and ΔΨm, indicating a distinct effect on core mitochondrial bioenergetics in fibroblasts that does not affect PPIX synthesis. This also indicates a potential shift in cellular energy homeostasis in Tspo−/− cells, which could affect multiple cellular events and responses. This shift in metabolic homeostasis could also explain gene expression differences in the heme biosynthetic pathway that were not only inconsistent between Tspo−/− bone marrow, fibroblasts, and MA-10 cells but also did not result in changes to PPIX production. In Tspo−/− primary microglia, a similar decrease in mitochondrial OCR was reported (10). Deletion of TSPO in Drosophila melanogaster (dTSPO−/−) also resulted in a decrease in mitochondrial OCR and oxidative phosphorylation complex activities (61). However, OCR changes were not observed in Tspo−/− mitochondria isolated from murine hepatocytes (16), suggesting that cell type-specific bioenergetics and other properties affected by the presence/absence or level of TSPO expression could influence observations.

Treatment of fibroblasts with PK11195 did not affect ALA to PPIX bioconversion. However, use of PK11195 decreased ΔΨm in both Tspo+/+/ and Tspo−/− cells, suggesting that at least part of the effects observed using this agent is not mediated by TSPO. This observation indicates that some of the actions of PK11195 and other TSPO-binding drugs that have been linked to several seemingly disparate functions, without a physiological explanation (reviewed in Ref. 3 and 4), may be due to off target effects. Early studies that utilized TSPO-binding drugs, PK11195 and Ro5-4864 (4'-chlorodiazepam) demonstrated that they could induce a dose-dependent decrease in OCR (62) and inhibit mitochondrial respiratory control (63), similar to that observed in Tspo−/− cells. However, given our results using PK11195 in Tspo−/− cells, it is clear that specificity of TSPO binding and off target effects may preclude the ability to interpret results from pharmacological studies.

In conclusion, our results suggest that despite its reported PPIX binding property, MmTSPO does not have an effect on erythropoiesis, heme biosynthesis, bioconversion of ALA to PPIX, or in porphyrin-mediated phototoxic cell death. Because TSPO is highly expressed in a variety of cancers, the lack of functional definition for TSPO does not preclude it from being a target for localizing photosensitizing agents as in PDT. However, meaningful interpretation of pharmacological effects
TSPO and Porphyrins

observed when using TSPO-binding drugs requires careful consideration of the limitations.

Author Contributions—V. S. and A. H. Z. conceived and designed the project. A. H. Z. performed most experiments, analyzed data, and interpreted results. L. N. T. performed experiments on mitochondrial bioenergetics, C. M. optimized the method and supervised mitochondrial membrane potential experiments. A. H. Z. and V. S. organized the figures and prepared the manuscript.

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Note Added in Proof—In the original version of the manuscript that was published as a JBC Paper in Press, panels in Fig. 3 and Fig. 4 reporting heme and PPIX values in the y axis were in error due to a miscalculation of coordinates and scale. The correct y axis labels and values for heme and PPIX are now presented in the revised Fig. 3 and Fig. 4. This correction does not change interpretation of results or the conclusions.

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