TOXICOLOGICAL SCIENCES **105(1)**, 44–50 (2008)
doi:10.1093/toxsci/kfn116
Advance Access publication June 11, 2008

Identification of Thioredoxin-2 as a Regulator of the Mitochondrial Permeability Transition

Min He,*,† Jiyang Cai,‡ Young-Mi Go,* Jennifer M. Johnson,*,† W. David Martin,§ Jason M. Hansen,¶ and Dean P. Jones*,[1]

*Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine; †Graduate Program in Nutrition Health Science; Emory University, Atlanta, Georgia 30322; ‡Vanderbilt Eye Institute, Vanderbilt University Medical Center, Nashville, Tennessee 37232; §Transgenic Mouse Facility; and ¶Division of Pulmonary, Allergy, Cystic Fibrosis and Sleep, Department of Pediatrics, Emory University, Atlanta, Georgia 30322

Received March 29, 2008; accepted June 5, 2008

Thioredoxin-2 (Trx2) is a multifunctional, mitochondria-specific protein, which inhibits cell death. The mitochondrial permeability transition (MPT) is a distinct mechanism for cell death activated by oxidants and linked to both necrotic and apoptotic morphologies. We studied mitochondria from Trx2 transgenic mice to determine whether Trx2 protects against oxidant-induced MPT. All experiments were performed in isolated mitochondria. Results showed that Trx2 protected against MPT induced by exogenously added peroxide. Unexpectedly, Trx2 also protected against the MPT induced by Ca^{2+} in the absence of added peroxide. The results indicate that in addition to protecting against oxidative stress, Trx2 is an endogenous regulator of the MPT.

Key Words: **transgenic mice; cell death mechanisms; apoptosis; necrosis; calcium.**

Thioredoxins are small proteins that perform a variety of functions dependent upon binding interactions and oxidoreductase activity catalyzed by a characteristic dithiol motif (Arner and Holmgren, 2000). Mammalian thioredoxin-2 (Trx2) is a mitochondrial form originally identified in pig heart (Bode stein and Follmann, 1991) and cloned and characterized from a rat heart cDNA library (Spyrou *et al*., 1997). Although thioredoxin-1 has been studied extensively, less information is available on Trx2.

Overexpression of human Trx2 (hTrx2) showed inhibition of peroxide-induced cell death in 143B osteosarcoma cells (Chen *et al*., 2002) and interaction with the mitochondrial respiratory chain to increase the mitochondrial membrane potential and increase resistance to etoposide-induced cell death in HEK-293 cells (Damdimopoulos *et al*., 2002). Mitochondria contain two thioredoxin-dependent peroxidases, peroxiredoxin-3 and -5 (Kang *et al*., 1998; Seo *et al*., 2000), and these activities provide a mechanism for Trx2-dependent antioxidant activity. A central role for Trx2 in mitochondria was further demonstrated in Trx2 knockout mice (Nonn *et al*., 2003b). Trx2 deficiency was embryonic lethal at gestational day 10.5 and embryos showed massive apoptosis. The timing coincided with the maturation of mitochondrial function and the transition in the embryo from anaerobic to aerobic metabolism (Nonn *et al*., 2003b). A direct interaction of Trx2 with Prx3 was recently demonstrated in a study using a dominant-negative form of Trx2 (Zhang *et al*., 2007a).

A mechanism for Trx2 control of oxidant-induced apoptosis was revealed by studies showing that reduced Trx2 binds to and inhibits apoptosis signal-regulating kinase-1 (ASK-1) (Zhang *et al*., 2004). Upon Trx2 oxidation and release, ASK-1 activates caspase-mediated apoptosis. Other studies showed that increased Trx2 blocked tumor necrosis factor-α (TNF-α)–induced apoptosis in HeLa cells, a process mediated by mitochondrial reactive oxygen species (ROS) (Hansen *et al*., 2006). In these cells, TNF-α treatment oxidized Trx2, and overexpression of Trx2 eliminated the mitochondrial ROS signal and blocked apoptosis (Hansen *et al*., 2006). Relevance of the antiapoptotic function of Trx2 to disease was recently demonstrated by targeted overexpression of Trx2 in vascular endothelium of mice (Zhang *et al*., 2007b). Results showed that Trx2 protected against vascular pathology in the apoE2-knockout mouse model for cardiovascular disease (Zhang *et al*., 2007b). Thus, the accumulated data strongly support a role for Trx2 in protecting against oxidant-induced apoptosis.

Considerable evidence also shows that mitochondria-mediated cell death occurs following activation of the mitochondrial permeability transition (MPT). The MPT is a high amplitude swelling of mitochondria (Hunter *et al*., 1976) triggered by the opening of a pore which allows rapid influx of ions and associated water (Bernardi, 1999). The MPT gained early interest as a central mechanism of cell death because mitochondrial swelling is a hallmark of necrosis (Imberti *et al*., 1993). Considerable research has also supports a role for the MPT in apoptosis (Nagahara *et al*., 2000; Precht *et al*., 2005; Skulachev, 1996; Zhang *et al*., 2008). The MPT pore is activated by Ca^{2+} in the presence of diverse agents, including those that induce oxidative stress (Gunter and Pfeiffer, 1990). Oxidants

[1] To whom correspondence should be addressed at Department of Medicine, 205 Whitehead Research Center, Emory University, Atlanta, GA 30322. Fax: (404) 712-2974. E-mail: dpjones@emory.edu.

disrupt cellular Ca^{2+} regulation, and increased cytoplasmic Ca^{2+} is an early event in oxidative stress preceding cell death (Orrenius *et al*., 2003).

In the present study, we developed transgenic (Tg) Trx2 mice to permit isolation of a sufficient quantity of high quality mitochondria to test whether Trx2 protected against activation of the MPT. The results from these studies show that isolated liver mitochondria from Trx2 Tg mice are protected against peroxide-induced MPT compared with wild-type (WT) littermate controls. Unexpectedly, an increased Ca^{2+} concentration was required to activate the MPT in the absence of added peroxide. The results demonstrate that in addition to protection against H_2O_2-induced mitochondrial damage and activation of apoptosis by ASK-1, Trx2 can inhibit the MPT, potentially providing a mechanism to protect against this form of mitochondria-mediated cell death.

MATERIALS AND METHODS

Trx2 Tg mouse. Tg mice were generated and maintained at the Emory University Transgenic Mouse Core Facility under approval of the Emory Institution Animal Care and Use Committee. Expression of the hTrx2 transgene was achieved by using the Cre-LoxP system (Kuhn and Torres, 2002). hTrx2 cDNA (Chen *et al*., 2002) was first cloned downstream of a chloramphenicol acetyltransferase (CAT) gene and a stop codon under the control of a chicken β-actin gene (CAG) promoter, which consists of a CAG promoter and a cytomegalovirus enhancer. The construct was microinjected into fertilized eggs isolated from C57/BL6 mice and transplanted into CD-1 female pseudopregnant mice. Germ line transmission was confirmed by PCR analyses using DNA isolated from mouse tail sampling and primers: 5′-GCC AAT CAG CTT CTT CAG GAA GGC-3′ and 5′-CAC CAT GGC TCA GCG ACT TCT TCT-3′. These primers did not identify endogenous mouse Trx2. PCR for Cre was performed with primers: 5′-ACC TGA AGA TGT TCG CGA TTA TCT-3′ and 5′-ACC GTC AGT ACG TGA GAT ATC TT-3′. The F1 mice carrying the hTrx2 gene were crossed with Protamine-Cre mice to remove the CAT gene and the stop codon so that the hTrx2 protein can be expressed. To normalize the genetic background for proteomic and MPT assays, the Tg mice were bred successively with 129SVev WT mice (Taconic) and maintained in this genetic background; for each experiment, comparisons were made between littermates. Mice were maintained under standard 12-h dark–light cycle at 20°C. Purina Rodent Chow 5001 diet and water were given *ad libitum*.

Immunoprecipitation, Western blot analysis, and activity measurements. Tissues were washed 3× with PBS and homogenized in RIPA lysis buffer containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.15M NaCl, 10mM sodium phosphate, pH 7.2, and protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Homogenates were centrifuged and supernatants were stored at −80°C for immunoprecipitation and Western blot analysis. Protein concentration was measured via the Bradford method (BioRad Life Science, Hercules, CA), and equal amounts of protein were used for Western blot analysis following SDS-polyacrylamide gel electrophoresis (PAGE) on 15% gels and blotting to nitrocellulose membrane. Membranes were probed with rabbit anti-Trx2 (Hansen *et al*., 2006), rabbit anti-cytochrome *c* (Cell Signaling Technology, Boston, MA), and mouse anti-V5 (Invitrogen, Carlsbad, CA) antibodies. A goat anti-GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used for verification of equal protein loading. An IRDye 800 conjugated affinity purified anti-rabbit or mouse IgG (Rockland Immunochemicals, Gilbersville, PA) or Alexa-Fluor-680-conjugated anti-goat (Invitrogen) was used as secondary antibody. Redox-Western blot analysis of Trx2 redox potential was performed as described (Hansen *et al*., 2006). Bands were visualized using an Odyssey scanner (Li-Cor Biosciences, Lincoln, NE) and quantified using the Odyssey 1.1 software (Li-Cor Biosciences, Lincoln, NE).

For immunoprecipitation, tissue extracts (400 μg protein) were mixed with 1.5 μl of anti-V5 antibody for 4 h at 4°C and then rotated with 50 μl of protein G magnetic beads (Qiagen, Valencia, CA) for 3 h at 4°C. Protein G beads with protein complex were denatured by loading buffer at 95°C for 5 min and run on 15% SDS-PAGE. Membranes were probed with a rabbit anti-Trx2 antibody.

Trx2 and thioredoxin reductase-2 (TrxR2) were measured in disrupted mitochondria using the insulin reduction assay (Sasada *et al*., 1999; Yang *et al*., 2004).

Mitochondria isolation. Mouse liver mitochondria were isolated following homogenization by differential centrifugation (Savage *et al*., 1991), and aliquots of the supernatant were kept for Western blot analysis as the cytosolic fraction. The pellets were either suspended in incubation buffer for MPT assay or RIPA buffer for Western blot analysis. For experiments to determine whether Trx2 was present in mitochondria from tissues other than liver, mitochondria were isolated from liver, heart, and skeletal muscle using the mitochondrial isolation kit (Sigma-Aldrich, St. Louis, MO) per the manufacturer's directions.

Submitochondrial localization of Trx2 by digitonin fractionation. Isolated mitochondria were treated with digitonin (0–4 mg/10 mg protein) for 20 min on ice and centrifuged at 9500 × g at 4°C for 15 min. The pellet was suspended in 150 μl of RIPA buffer, and aliquots of supernatant and pellet were stored at −80°C for subsequent Western blot analysis. Adenylate kinase (AK) was used as a marker for the intermembrane space with the criterion that release of Trx2 in parallel with AK would indicate an intermembrane space location, whereas release of AK at concentrations lower than that required for Trx2 would indicate matrix location (Das, 1981; Kuylenstierna *et al*., 1970). Heat shock protein-60 (HSP60) was used as a matrix marker and was measured by Western blot (mouse monoclonal, Santa Cruz with Alexa-Fluor-680 donkey-anti-mouse secondary antibody, Invitrogen).

MPT assay. Freshly isolated liver mitochondria from both WT and Trx2 Tg mice were suspended in incubation medium containing 250mM sucrose, 10mM (3-[N-morpholino]propanesulfonic acid]), 3mM K_2PO_4, and 5mM sodium succinate, pH 7.25. Mitochondria (300 μg protein) were suspended into incubation medium with varied concentrations of Ca^{2+} or *tert*-butylhydroperoxide (tBH) to give a total volume of 1 ml, and MPT was determined spectrophotometrically at 540 nm (Savage *et al*., 1991).

Ca^{2+} content of mitochondria. Mitochondrial Ca^{2+} was measured by the method of Savage *et al*. (1991). Aliquots of mitochondria incubated as above in the absence and presence of cyclosporine A (0.5μM) were taken at 0, 2, 4, 6, 8, 10, 20, and 30 min after addition of Ca^{2+} and were centrifuged for 1 min at 13,000 × g. The supernatant was completely removed and the mitochondrial pellet was treated with 10% perchloric acid and centrifuged to remove protein. Following neutralization with 1M potassium carbonate and removal of the precipitated potassium perchlorate, 20 μl was added to 50μM Fura-2 (Sigma) in 0.1M Tris (pH 8.0). Fluorescence was measured with excitation at 340 and 380 nm and emission at 510 nm; the ratio was used to calculate Ca^{2+} concentration relative to a standard curve.

Statistics. Data are expressed as mean ± SEM and analyzed by paired *t*-test, ANOVA with *post hoc* analysis by Student-Newman-Keuls test as appropriate.

RESULTS

Characteristics of Trx2 Tg Mice

To facilitate distinction between endogenous Trx2 and the transgene product, we incorporated a V5 epitope at the C-terminus of hTrx2 (Chen *et al*., 2002). hTrx2 differs from mouse Trx2 in two amino acids (human, mouse: I5V; M80I) (Damdimopoulos *et al*., 2002; Tanaka *et al*., 2002) in the mature mitochondrial form, and these differences are not

known to affect catalytic or binding activities. Fractionation of liver tissue into cytosolic and mitochondrial fractions showed that Trx2 was present in the mitochondria but not in the cytoplasm (Fig. 1A). Mitochondrial fractions from heart and skeletal muscle also showed specific localization of Trx2 to the mitochondria and not cytoplasm (not shown). Activity measurements in isolated mitochondria with the insulin reduction assay showed that Trx2 activity was increased 63% in Tg mitochondria relative to WT littermate controls, whereas there was no significant change in TrxR2 activity (Fig. 1B). No detectable differences in redox potential of Trx2, as measured by the redox-Western blotting (Hansen *et al*., 2006), or mitochondrial membrane potential, as estimated by tetramethyl rhodamine methyl ester (Savage *et al*., 1991), were apparent. Cytochrome *c* content by Western blot analysis was not significantly different in liver extracts or isolated mitochondria from Tg and WT mice (data not shown).

The location of Trx2 within the mitochondria was evaluated using digitonin fractionation of isolated liver mitochondria. Results showed that at low concentrations of digitonin, Trx2 was retained in the pellet, but the intermembrane space marker AK was released into the supernatant fraction (Fig. 2). At higher digitonin concentrations, Trx2 was released into the supernatant fraction (outer membrane and intermembrane space proteins) over the same digitonin concentration range which released the matrix protein, HSP60 (Fig. 2). These results show that Trx2 is principally, if not exclusively, present in the matrix space or complexed with inner membrane-associated proteins.

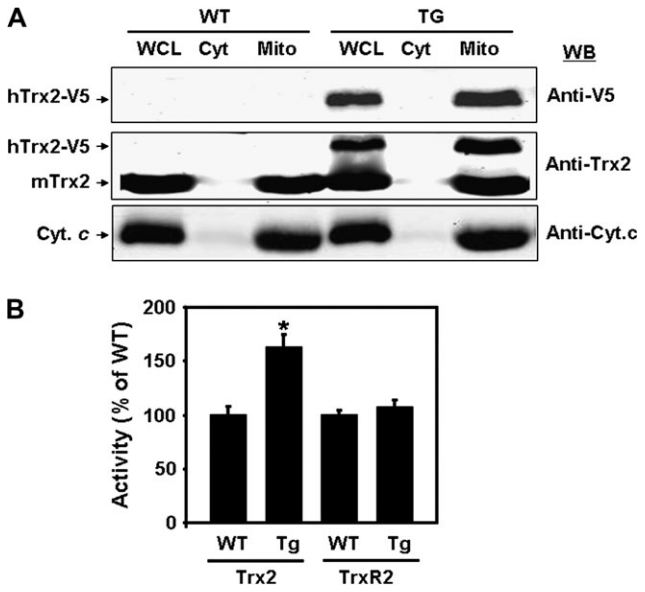

FIG. 1. Mitochondrial localization of the hTrx2 and increase in mitochondrial Trx2 activity. (A) To examine distribution of hTrx2-V5, liver homogenates (WCL: whole cell lysate) were separated into mitochondrial (Mito) and cytosolic (Cyt) fractions by differential centrifugation, resolved by SDS-PAGE and analyzed by Western blotting with antibodies to the V5 epitope to detect hTrx2-V5, Trx2 to detect both hTrx2-V5 and endogenous mouse Trx2 (mTrx2), and cytochrome *c* (Cyt *c*) as a marker for mitochondria. Data are representative of three separate experiments. Identical results were obtained for heart and skeletal muscle fractionation studies. (B) Mitochondrial Trx2 and TrxR2 activities were measured with the NADPH-insulin reduction assay for liver mitochondria with Tg values being expressed relative to a paired WT littermate. Data are from duplicate assays on three pairs of mitochondrial preparations for Trx2 and for duplicate assays on two pairs of mitochondrial preparations for TrxR2. *Significantly different from WT value, $p < 0.05$.

Trx2 Protected against Ca^{2+}-Induced MPT

Experiments were performed to determine whether increased Trx2 altered the MPT in mitochondria isolated from livers of Trx2 Tg mice compared with those from WT littermates. For each experiment, a sibling pair consisting of an hTrx2 Tg and a WT animal was selected, and mitochondria were prepared, diluted to equivalent protein concentrations (0.3 mg/ml) and analyzed in parallel. In the presence of 3mM inorganic phosphate and 80μM Ca^{2+}, mitochondria from both Tg and WT animals showed characteristic MPT responses and both were sensitive to cyclosporine A (CsA; Fig. 3A). The maximal ΔA_{540} was not significantly different for liver mitochondria from Tg and WT mice when exposed to this Ca^{2+} overload condition (0.35 ± 0.03 and 0.34 ± 0.02; $p = 0.71$, $n = 4$). This result indicated that there was no substantial difference in the volume or extent of swelling of the mitochondria due to the transgene. To determine whether there was a difference in Ca^{2+} loading capacity, Tg and WT littermate control mitochondria were incubated as in Figure 3A and assayed for mitochondrial Ca^{2+} content. In the absence of CsA, maximal Ca^{2+} loading occurred at 4–6 min and was not significantly different between Tg and WT mitochondria (131 ± 7 and 139 ± 15 nmol/mg protein, respectively). In the presence of CsA, the extent of Ca^{2+} loading was similar to that without CsA and was not significantly different between Tg and WT mitochondria (140 ± 3

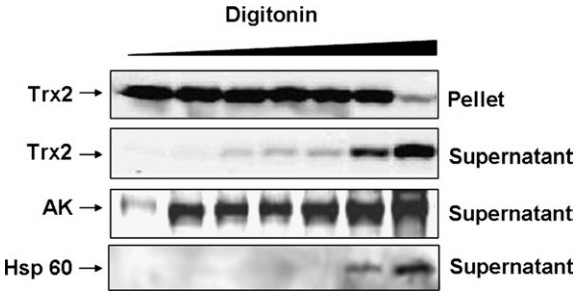

FIG. 2. Submitochondrial compartmentation of Trx2. Experiments were performed in which increasing concentrations of digitonin were added to isolated mitochondria. Mitochondria were pelleted by centrifugation to allow detection of released proteins in the supernatant. Western blotting was used to determine whether Trx2 was present in the intermembrane space or in the matrix space. Trx2 was released (top and middle panels) only at high concentrations of digitonin, whereas the intermembrane space marker AK (bottom panel) was released at low concentration of digitonin. Data from a single experiment which is representative of data from three separate mitochondrial preparations.

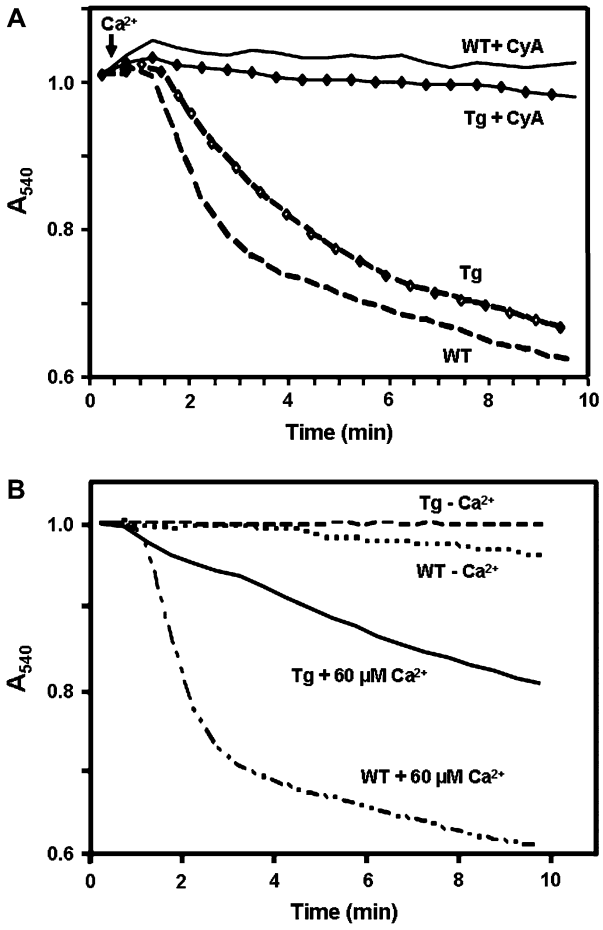

FIG. 3. The MPT in liver mitochondria from Trx2 Tg mice. (A) The MPT in Tg mitochondria was similar to that observed in WT littermates and was inhibited by cyclosporine A (CsA). Mitochondria were suspended in media containing 3mM inorganic phosphate, 5mM sodium succinate and 80μM Ca^{2+} at 20° and activation of the MPT was monitored at 540 nm. Data are representative of results from four pairs of liver mitochondria and two pairs of heart mitochondria. (B) Mitochondria from Trx2 mice were resistant to Ca^{2+}-dependent activation of the MPT. Although the MPT was similar in mitochondria from Tg and WT littermates when 80μM Ca^{2+} was present, at 60μM and lower concentrations, the MPT was decreased. Conditions for B were identical to A except that Ca^{2+} was lower, and this resulted in delayed time course for MPT. Data are representative of experiments from four littermate pairs of Tg and WT mice.

and 139 ± 4 nmol/mg protein, respectively). Previous research showed that overexpression of Trx2 in cells increased mitochondrial membrane potential (Damdimopoulos *et al.*, 2002), but we did not find any apparent difference between Tg and WT mitochondria. Although these data do not provide information on rates of Ca^{2+} uptake, they suggest that there is a similar membrane potential driving force and a similar Ca^{2+} loading capacity.

The oxidant-induced MPT is dependent upon Ca^{2+}, so initial experiments were designed to obtain an appropriate Ca^{2+} concentration which would be sufficient to quantify the oxidant sensitivity but did not rapidly induce the MPT alone. Concentration-dependence studies showed that at Ca^{2+} concentrations of 20μM or lower, the MPT was not observable within 15 min in either Tg or WT mitochondria. However, at higher concentrations, the MPT was activated in both WT and Tg mitochondria, with the Tg mitochondria showing a resistance to Ca^{2+} (Fig. 3B). To obtain quantitative comparisons, we measured the time required to obtain 20% of the maximal MPT response. With 60μM Ca^{2+} respective times for WT and Tg mitochondria were 1.8 ± 0.3 min for WT and 4.2 ± 0.3 min for Tg. Comparable results were obtained with mitochondrial preparations from hearts of Tg and WT mice (data not shown). Thus, the results show that the hTrx2 significantly delayed activation of the MPT in response to Ca^{2+}.

Trx2 Protected against Oxidant-Induced MPT

To determine whether Trx2 protected against oxidant-induced MPT, 20μM Ca^{2+} was selected to allow sensitive detection of the effects of increasing concentrations of tBH on the MPT. Pilot studies were done with a range of Ca^{2+} concentrations and provided results which qualitatively agreed with the data described below. At Ca^{2+} concentrations below 20μM, less activation occurred and less stimulation was seen with tBH. At higher concentrations, greater activation occurred and this was stimulated by peroxide. Consequently, there was a relatively narrow range of conditions which were useful.

For each respective Tg and WT pair, the time courses differed with the characteristic that Tg mitochondria had a slower time course of response at all tBH concentrations. For example, with 20μM Ca^{2+} and 3mM inorganic phosphate, 10% of the maximal MPT absorbance change occurred at 16.5 ± 1.6 min in WT and 18.9 ± 1.1 min in Tg mitochondria. The addition of 12.5μM tBH decreased the time to induce a 10% change to 14.0 ± 1.3 min in WT and 18.1 ± 2.0 min in Tg mitochondria. At 25μM, 10% effect occurred at 12.8 ± 2.3 min in WT and 18.0 ± 2.6 min in Tg mitochondria. At 50μM, 10% effect occurred at 8.5 ± 1.5 min in WT and 11.9 ± 0.6 min in Tg mitochondria.

To graphically illustrate the difference in sensitivity, the percentage of maximal MPT was determined at 20 min (Fig. 4). For this purpose, the maximal MPT was taken as the absorbance change occurring with 80μM Ca^{2+}, a concentration above which no further change is observed Liver mitochondria from Tg and WT mice were assayed in the presence of 20μM Ca^{2+} and 3mM inorganic phosphate, with additions of 12.5, 25, or 50μM tBH. Results showed that data for respective Tg and WT pairs were significantly different at all tBH concentrations. Consequently, the data show that hTrx2 expression increased the resistance of mitochondria to oxidant-induced MPT. The decreased activation and apparent lack of dose response for the tBH treatment of hTrx2 mitochondria were consistent with two effects, a resistance of the MPT to activation by Ca^{2+} and a resistance of the Ca^{2+}-dependent MPT to tBH.

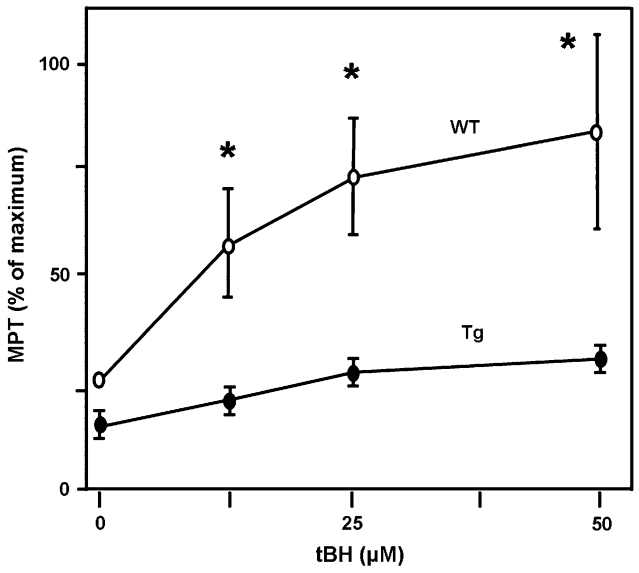

FIG. 4. Trx2 protection against oxidant-induced MPT. To determine whether Trx2 protected against oxidant-induced toxicity, liver mitochondria from Tg and WT mice were assayed for the MPT in the presence of 20μM Ca^{2+} and 3mM inorganic phosphate, with additions of 0, 12.5, 25, or 50μM tBH. Data are presented as the percentage of the maximal MPT response obtained under the conditions indicated at 20 min. Results are means ± SEM from experiments on four pairs of Tg and WT littermates. Data for respective Tg and WT pairs were significantly different at all tBH concentrations. Data were analyzed by ANOVA with *post hoc* analysis by Student-Newman-Keuls test. *Significant at $p < 0.05$.

DISCUSSION

In recent years, a major shift in oxidative stress research has occurred with the recognition that H_2O_2 and redox-sensitive cysteines of proteins function in cell signaling and control. This has resulted in a redefinition of oxidative stress to include disruption of redox signaling and control along with macromolecular damage as toxicologic consequences (Jones, 2006). The implications of this redefinition for toxicologic mechanisms involving mitochondria are important because of the integration of redox signaling and control with other central mechanisms, including H_2O_2 signaling, Ca^{2+} signaling and kinase signaling, as activators of cell death. In this context, the finding that oxidation of Trx2 can result in activation of Ask-1 as an apoptosis mechanism (Zhang *et al.*, 2004) raised the question whether earlier results implicating the MPT were misinterpreted. The current results show explicitly that the content of Trx2 in mitochondria protects against activation of the MPT. Although this does not establish involvement of the MPT in any specific cell toxicity model, it shows that in addition to inhibition of Ask-1, Trx2 also protects against the MPT. Thus, the results show that one cannot simply discount previous findings of oxidative effects on the MPT due to the discovery of the Ask-1 mechanism. Whether the function of Trx2 in blocking two different cell death pathways represents a coordinated control of death mechanisms or alternative mechanisms that are differentially expressed in cells is not known; however, the results highlight the need to discriminate between MPT- and Ask-1–mediated mechanisms in cell toxicity research.

Accumulating data show that Trx2 can protect against toxicity by multiple mechanisms. Reduced Trx2 binds to ASK-1 and inhibits its activity, thereby protecting against apoptosis (Zhang *et al.*, 2004). Trx2 interacts with Prx3 (Zhang *et al.*, 2007a) suggesting that Trx2 supports peroxide reduction in mitochondria. In support of this conclusion, overexpression of Trx2 blocked TNF-α–induced ROS production (Zhang *et al.*, 2007a). Overexpression of Prx5, another thioredoxin-dependent peroxidase found in mitochondria, also protected against peroxide-induced mitochondrial DNA damage (Banmeyer *et al.*, 2004). Thus, the present data add to the accumulating evidence that Trx2 provides central protection against mitochondrial mechanisms of toxicity.

During recent years, it has become increasingly apparent that multiple mechanisms exist for mitochondria-mediated cell death (Dietze *et al.*, 2001; Nagahara *et al.*, 2000; Precht *et al.*, 2005). These include different pathways for release of cytochrome *c* and activation of caspases, as well as activation of mitochondrial ASK-1 (Dietze *et al.*, 2001; Lim *et al.*, 2008; Zhang *et al.*, 2008). A number of studies implicate the MPT in activation of cell death by apoptosis and necrosis (Lim *et al.*, 2008; Precht *et al.*, 2005; Tsujimoto *et al.*, 2006), and Trx2 has been shown to protect against cell death in cell culture experiments (Chen *et al.*, 2002; Hansen *et al.*, 2006). *In vivo*, knockout mice are embryonic lethal, showing massive apoptosis at a time of mitochondrial maturation (Nonn *et al.*, 2003a). In addition, conditionally deficient chicken DT40 B-cells were found to undergo apoptosis upon repression of the transgene (Tanaka *et al.*, 2002). Similar studies are needed to determine whether mitochondria in cells from the Tg mice are protected against oxidant-induced MPT and whether mice with conditional repression of Trx2 have increased susceptibility. Available evidence shows that overexpression of Trx2 protects against oxidant-, etoposide-, and TNF-α–induced cell death (Chen *et al.*, 2002; Damdimopoulos *et al.*, 2002; Hansen *et al.*, 2006). Whether any of these mechanisms are specifically linked to prevention of the MPT is not known, although HeLa cells overexpressing Trx2 are protected against loss of the mitochondrial membrane potential dye, JC-1 (J. M. Hansen, unpublished observation).

The *in vitro* swelling assay is a highly artificial system to study the MPT but it is a standardized protocol for measuring MPT in isolated mitochondria. However, cellular studies on Trx2 protection against oxidant-induced cell death do not show whether Trx2 has an effect on the MPT as opposed to activation of the Ask-1 pathway. Thus, even though the *in vitro* assay is only a model for the physiologic function, it provides direct evidence that Trx2 affects the MPT without contribution from non-mitochondrial factors. Many factors induce the MPT, including oxidative stress (Bernardi, 1999). The MPT can be

facilitated by thiol oxidants such as diamide and phenylarsine oxide and inhibited by antioxidants, such as dithiothreitol (Fagian *et al*., 1990; Lenartowicz *et al*., 1991). Due to the reversibility and oxidant/reductant sensitivity, proposed mechanisms include redox-sensitive cysteine residue regulation (Halestrap and Brennerb, 2003). Two distinct mechanisms are implicated in thiol regulation of the MPT (Chernyak and Bernardi, 1996; Costantini *et al*., 1996, 2000). One is sensitive to oxidants and the other to the redox state of matrix $NADP^+$ (nicotinamide adenine dinucleotide phosphate) on the MPT pore. However, many mitochondrial proteins contain redox-sensitive thiols, and the present data do not provide any clarification concerning which of these could be responsible for the redox sensitivity of the MPT. Redox proteomic methods (Fratelli *et al*., 2002; Leichert *et al*., in press) provide important new opportunities to identify such mechanistic links.

Furthermore, whether Trx2 directly interacts with components of the pore or only indirectly regulates the pore remains uncertain. Trx2 overexpression in 143B osteosarcoma cells did not increase the rate of exogenous peroxide elimination (Chen *et al*., 2002), but these measurements may not reflect the amount of ROS elimination within the mitochondria. Other studies show increased ROS in cells with decreased Trx2 (Tanaka *et al*., 2002) and decreased ROS in TNF-α-treated cells overexpressing Trx2 (Hansen *et al*., 2006). Thus, Trx2 could potentially regulate the MPT either directly by interacting with MPT pore components or indirectly by altering the mitochondrial concentrations of oxidants which affect the function of the MPT pore complex.

In summary, Trx2 is present in the matrix of mitochondria and protects mitochondria from calcium- and oxidant-induced MPT. This supports the interpretation that Trx2 can protect against cell death by mechanisms mediated by the MPT. Further study is required to identify the protein targets and outline specifics of this potential mechanism.

FUNDING

National Institutes of Health (ES009047) to D. J., and (ES014668) to J. C.

REFERENCES

Arner, E. S., and Holmgren, A. (2000). Physiological functions of thioredoxin and thioredoxin reductase. *Eur. J. Biochem.* **267,** 6102–6109.

Banmeyer, I., Marchand, C., Verhaeghe, C., Vucic, B., Rees, J. F., and Knoops, B. (2004). Overexpression of human peroxiredoxin 5 in subcellular compartments of Chinese hamster ovary cells: Effects on cytotoxicity and DNA damage caused by peroxides. *Free Radic. Biol. Med.* **36,** 65–77.

Bernardi, P. (1999). Mitochondrial transport of cations: Channels, exchangers, and permeability transition. *Physiol. Rev.* **79,** 1127–1155.

Bode stein, J., and Follmann, H. (1991). Characterization of two thioredoxins in pig heart including a new mitochondrial protein. *Z. Naturforsch. C* **46,** 270–279.

Chen, Y., Cai, J., Murphy, T. J., and Jones, D. P. (2002). Overexpressed human mitochondrial thioredoxin confers resistance to oxidant-induced apoptosis in human osteosarcoma cells. *J. Biol. Chem.* **277,** 33242–33248.

Chernyak, B. V., and Bernardi, P. (1996). The mitochondrial permeability transition pore is modulated by oxidative agents through both pyridine nucleotides and glutathione at two separate sites. *Eur. J. Biochem.* **238,** 623–630.

Costantini, P., Belzacq, A. S., Vieira, H. L., Larochette, N., de Pablo, M. A., Zamzami, N., Susin, S. A., Brenner, C., and Kroemer, G. (2000). Oxidation of a critical thiol residue of the adenine nucleotide translocator enforces Bcl-2-independent permeability transition pore opening and apoptosis. *Oncogene* **19,** 307–314.

Costantini, P., Chernyak, B. V., Petronilli, V., and Bernardi, P. (1996). Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites. *J. Biol. Chem.* **271,** 6746–6751.

Damdimopoulos, A. E., Miranda-Vizuete, A., Pelto-Huikko, M., Gustafsson, J. A., and Spyrou, G. (2002). Human mitochondrial thioredoxin. Involvement in mitochondrial membrane potential and cell death. *J. Biol. Chem.* **277,** 33249–33257.

Das, S. K. (1981). The submitochondrial localization adenylate kinase: An enzymatic marker for the inner surface of the outer membrane of lung mitochondria in guinea pig. *Biochem. Biophys. Res. Commun.* **103,** 1145–1148.

Dietze, E. C., Caldwell, L. E., Grupin, S. L., Mancini, M., and Seewaldt, V. L. (2001). Tamoxifen but not 4-hydroxytamoxifen initiates apoptosis in p53(-) normal human mammary epithelial cells by inducing mitochondrial depolarization. *J. Biol. Chem.* **276,** 5384–5394.

Fagian, M. M., Pereira-da-Silva, L., Martins, I. S., and Vercesi, A. E. (1990). Membrane protein thiol cross-linking associated with the permeabilization of the inner mitochondrial membrane by Ca2+ plus prooxidants. *J. Biol. Chem.* **265,** 19955–19960.

Fratelli, M., Demol, H., Puype, M., Casagrande, S., Eberini, I., Salmona, M., Mengozzi, M., Duffieux, F., Miclet, E., Bachi, A., *et al*. (2002). Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 3505–3510.

Gunter, T. E., and Pfeiffer, D. R. (1990). Mechanisms by which mitochondria transport calcium. *Am. J. Physiol.* **258,** C755–C786.

Halestrap, A. P., and Brennerb, C. (2003). The adenine nucleotide translocase: A central component of the mitochondrial permeability transition pore and key player in cell death. *Curr. Med. Chem.* **10,** 1507–1525.

Hansen, J. M., Zhang, H., and Jones, D. P. (2006). Mitochondrial thioredoxin-2 has a key role in determining tumor necrosis factor-alpha-induced reactive oxygen species generation, NF-kappaB activation, and apoptosis. *Toxicol. Sci.* **91,** 643–650.

Hunter, D. R., Haworth, R. A., and Southard, J. H. (1976). Relationship between configuration, function, and permeability in calcium-treated mitochondria. *J. Biol. Chem.* **251,** 5069–5077.

Imberti, R., Nieminen, A. L., Herman, B., and Lemasters, J. J. (1993). Mitochondrial and glycolytic dysfunction in lethal injury to hepatocytes by t-butylhydroperoxide: Protection by fructose, cyclosporin A and trifluoperazine. *J. Pharmacol. Exp. Ther.* **265,** 392–400.

Jones, D. P. (2006). Redefining oxidative stress. *Antioxid. Redox Signal.* **8,** 1865–1879.

Kang, S. W., Chae, H. Z., Seo, M. S., Kim, K., Baines, I. C., and Rhee, S. G. (1998). Mammalian peroxiredoxin isoforms can reduce hydrogen peroxide generated in response to growth factors and tumor necrosis factor-alpha. *J. Biol. Chem.* **273,** 6297–6302.

Kuhn, R., and Torres, R. M. (2002). Cre/loxP recombination system and gene targeting. *Methods Mol. Biol.* **180,** 175–204.

Kuylenstierna, B., Nicholls, D. G., Hovmoller, S., and Ernster, L. (1970). Effect of trypsin on mitochondrial and microsomal enzymes. *Eur. J. Biochem.* **12,** 419–426.

Leichert, L. I., Gehrke, F., Gudiseva, H. V., Blackwell, T., Ilbert, M., Walker, A. K., Strahler, J. R., Andrews, P. C., and Jakob, U. (2008). Quantifying changes in the thiol redox proteome upon oxidative stress in vivo. *Proc. Natl. Acad. Sci. U. S. A* **105**(24), 8197–8202.

Lenartowicz, E., Bernardi, P., and Azzone, G. F. (1991). Phenylarsine oxide induces the cyclosporin A-sensitive membrane permeability transition in rat liver mitochondria. *J. Bioenerg. Biomembr.* **23,** 679–688.

Lim, P. L., Liu, J., Go, M. L., and Boelsterli, U. A. (2008). The mitochondrial superoxide/thioredoxin-2/Ask1 signaling pathway is critically involved in troglitazone-induced cell injury to human hepatocytes. *Toxicol. Sci.* **101,** 341–349.

Nagahara, Y., Ikekita, M., and Shinomiya, T. (2000). Immunosuppressant FTY720 induces apoptosis by direct induction of permeability transition and release of cytochrome c from mitochondria. *J. Immunol.* **165,** 3250–3259.

Nonn, L., Berggren, M., and Powis, G. (2003a). Increased expression of mitochondrial peroxiredoxin-3 (thioredoxin peroxidase-2) protects cancer cells against hypoxia and drug-induced hydrogen peroxide-dependent apoptosis. *Mol. Cancer Res.* **1,** 682–689.

Nonn, L., Williams, R. R., Erickson, R. P., and Powis, G. (2003b). The absence of mitochondrial thioredoxin 2 causes massive apoptosis, exencephaly, and early embryonic lethality in homozygous mice. *Mol. Cell. Biol.* **23,** 916–922.

Orrenius, S., Zhivotovsky, B., and Nicotera, P. (2003). Regulation of cell death: The calcium-apoptosis link. *Nat Rev Mol Cell Biol.* **4,** 552–565.

Precht, T. A., Phelps, R. A., Linseman, D. A., Butts, B. D., Le, S. S., Laessig, T. A., Bouchard, R. J., and Heidenreich, A. (2005). The permeability transition pore triggers Bax translocation to mitochondria during neuronal apoptosis. *Cell Death Differ.* **12,** 255–265.

Sasada, T., Nakamura, H., Ueda, S., Sato, N., Kitaoka, Y., Gon, Y., Takabayashi, A., Spyrou, G., Holmgren, A., and Yodoi, J. (1999). Possible involvement of thioredoxin reductase as well as thioredoxin in cellular sensitivity to cis-diamminedichloroplatinum (II). *Free Radic. Biol. Med.* **27,** 504–514.

Savage, M. K., Jones, D. P., and Reed, D. J. (1991). Calcium- and phosphate-dependent release and loading of glutathione by liver mitochondria. *Arch. Biochem. Biophys.* **290,** 51–56.

Seo, M. S., Kang, S. W., Kim, K., Baines, I. C., Lee, T. H., and Rhee, S. G. (2000). Identification of a new type of mammalian peroxiredoxin that forms an intramolecular disulfide as a reaction intermediate. *J. Biol. Chem.* **275,** 20346–20354.

Skulachev, V. P. (1996). Why are mitochondria involved in apoptosis? Permeability transition pores and apoptosis as selective mechanisms to eliminate superoxide-producing mitochondria and cell. *FEBS Lett.* **397,** 7–10.

Spyrou, G., Enmark, E., Miranda-Vizuete, A., and Gustafsson, J. (1997). Cloning and expression of a novel mammalian thioredoxin. *J. Biol. Chem.* **272,** 2936–2941.

Tanaka, T., Hosoi, F., Yamaguchi-Iwai, Y., Nakamura, H., Masutani, H., Ueda, S., Nishiyama, A., Takeda, S., Wada, H., Spyrou, G., *et al.* (2002). Thioredoxin-2 (TRX-2) is an essential gene regulating mitochondria-dependent apoptosis. *EMBO J.* **21,** 1695–1703.

Tsujimoto, Y., Nakagawa, T., and Shimizu, S. (2006). Mitochondrial membrane permeability transition and cell death. *Biochim. Biophys. Acta* **1757,** 1297–1300.

Yang, X., Wu, X., Choi, Y. E., Kern, J. C., and Kehrer, J. P. (2004). Effect of acrolein and glutathione depleting agents on thioredoxin. *Toxicology* **204,** 209–218.

Zhang, D., Lu, C., Whiteman, M., Chance, B., and Armstrong, J. S. (2008). The mitochondrial permeability transition regulates cytochrome c release for apoptosis during endoplasmic reticulum stress by remodeling the cristae junction. *J. Biol. Chem.* **283,** 3476–3486.

Zhang, H., Go, Y. M., and Jones, D. P. (2007a). Mitochondrial thioredoxin-2/peroxiredoxin-3 system functions in parallel with mitochondrial GSH system in protection against oxidative stress. *Arch. Biochem. Biophys.* **465,** 119–126.

Zhang, H., Luo, Y., Zhang, W., He, Y., Dai, S., Zhang, R., Huang, Y., Bernatchez, P., Giordano, F. J., Shadel, G., *et al.* (2007b). Endothelial-specific expression of mitochondrial thioredoxin improves endothelial cell function and reduces atherosclerotic lesions. *Am. J. Pathol.* **170,** 1108–1120.

Zhang, R., Al-Lamki, R., Bai, L., Streb, J. W., Miano, J. M., Bradley, J., and Min, W. (2004). Thioredoxin-2 inhibits mitochondria-located ASK1-mediated apoptosis in a JNK-independent manner. *Circ. Res.* **94,** 1483–1491.