Mitochondrial Thioredoxin Reductase Is Essential for Early Postischemic Myocardial Protection

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Background—Excessive formation of reactive oxygen species contributes to tissue injury and functional deterioration after myocardial ischemia/reperfusion. Especially, mitochondrial reactive oxygen species are capable of opening the mitochondrial permeability transition pore, a harmful event in cardiac ischemia/reperfusion. Thioredoxins are key players in the cardiac defense against oxidative stress. Mutations in the mitochondrial thioredoxin reductase (thioredoxin reductase-2, Txnrd2) gene have been recently identified to cause dilated cardiomyopathy in patients. Here, we investigated whether mitochondrial thioredoxin reductase is protective against myocardial ischemia/reperfusion injury.

Methods and Results—In mice, α-MHC-restricted Cre-mediated Txnrd2 deficiency, induced by tamoxifen (Txnrd2−/−ic), aggravated systolic dysfunction and cardiomyocyte cell death after ischemia (90 minutes) and reperfusion (24 hours). Txnrd2−/−ic was accompanied by a loss of mitochondrial integrity and function, which was resolved on pretreatment with the reactive oxygen species scavenger N-acetylcysteine and the mitochondrial permeability transition pore blocker cyclosporin A. Likewise, Txnrd2 deletion in embryonic endothelial precursor cells and embryonic stem cell-derived cardiomyocytes, as well as introduction of Txnrd2-shRNA into adult HL-1 cardiomyocytes, increased cell death on hypoxia and reoxygenation, unless N-acetylcysteine was coinstantiated.

Conclusions—We report that Txnrd2 exerts a crucial function during postischemic reperfusion via thiol regeneration. The efficacy of cyclosporin A in cardiac Txnrd2 deficiency may indicate a role for Txnrd2 in reducing mitochondrial reactive oxygen species, thereby preventing opening of the mitochondrial permeability transition pore. (Circulation. 2011;124:2892-2902.)

Key Words: reactive oxygen species • ischemia reperfusion injury • infarct size

Excessive formation of reactive oxygen species (ROS) during postischemic reperfusion significantly contributes to tissue injury and functional deterioration of the heart. Cardiomyocytes contain a high amount of mitochondria to provide energy equivalents for generating force. However, mitochondria are the major site of generation of an oxygen radical burst on reperfusion in vitro and in vivo. Under normoxic conditions, in vitro ROS formation occurs at a rate of approximately 1% to 2% of oxygen consumption. In contrast, posthypoxic reoxygenation results in massive mitochondrial superoxide formation, triggering subsequent formation of hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (OH•). Myocardial ischemia and reperfusion (I/R) injury has been shown to be attenuated by endogenous ROS scavenging systems, such as mitochondrial manganese superoxide dismutase, detoxifying O$_2$ and catalase or glutathione peroxidase, which inactivate H$_2$O$_2$.

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Less well studied is the thioredoxin / thioredoxin reductase system. Thioredoxins work through a –Cys-Gly-Pro-Cys–active site and supply electrons for the reduction of a wide variety of substrates, including peroxiredoxins, which are critically in-
volved in ROS scavenging. Thioredoxin-1 (Txn1) and its corresponding reductase, thioredoxin reductase-1 (Txnrd1), are primarily localized in the cytosol, whereas thioredoxin-2 (Txn2) and its reductase, thioredoxin reductase-2 (Txnrd2), are both expressed in mitochondria. A third form, thioredoxin reductase-3, is mainly expressed in testis, and thus, has not been included in this study.

Txn1 has been demonstrated to participate in cardiac ROS scavenging and in limiting postischemic apoptosis and infarct size. By contrast, much less is known about the cardio-protective role of Txn2 and Txnrd2. The subcellular localization in mitochondria fueled speculation that Txn2 is central in the regulation of mitochondrial ROS in cardiomyocytes, given a solid 30% contribution of mitochondria to the cardiomyocyte cell volume. A potentially crucial role of Txn2 in modulating cardiac hypertrophy on stimulation by angiotensin 2 surfaced recently, consistent with the notion that the hypertrophy-driving O2 and H2O2 force is controlled by Txn2.

Unfortunately, analysis of Txnrd1 and Txnrd2, which antagonize the myocardial ROS burst at the onset of postischemic reperfusion, was hampered by embryonic lethality of ubiquitous deletion of either reductase. To overcome this obstacle, we were able to generate mice with cardiomyocyte-specific Txnrd1 deletion, which were born at the normal Mendelian ratio and proved to be fully viable. In contrast, constitutive cardiomyocyte-restricted Txnrd2 deletion caused early postnatal death, prompting the generation of tamoxifen-inducible cardiac-specific Txnrd2-deficient mice. Taking advantage of cardiomyocyte-specific Txnrd1- or Txnrd2-deficient mice, we were able to define the role of mitochondrial versus cytosolic thioredoxin reductases in the scenario of I/R injury for the first time.

**Materials and Methods**

**Heart-Specific Disruption of Txnrd1 and Txnrd2**

All animal experiments were approved by the Bavarian Animal Care and Use Committee and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Approved Institution #A5637-01), Heart-specific inactivation of Txnrd1 was achieved by using the MLC2a-Cre transgenic mouse line, as described. Tomato mice containing a fluorescence switch before (red, tomato) and after (green, eGFP) Cre activation (online-only Data Supplement Figure 1A) were kindly provided by Ralf Adams, Muenster, Germany. Mice overexpressing human thioredoxin-2 (TG1-2+2+) were created by Dr Jiyang Cai in collaboration with Dr. Dean P. Jones, Emory University, Atlanta, Georgia.

Cardiac tissue-specific disruption of Txnrd2 was achieved by crossing Txnrd2flox/l-knockout with a heart-specific tamoxifen-inducible Cre recombinase-expressing strain and feeding adult mice for 4 weeks with chow containing tamoxifen citrate. Cre recombinase-expressing strain and feeding adult mice for 4 weeks with chow containing tamoxifen citrate (= Txnrd2-/ic), followed by 4 weeks of standard diet. 4 weeks of tamoxifen citrate chow were followed by 2 weeks of standard diet.

**Ischemia and Reperfusion**

Ischemia of the heart was induced for 90 minutes by ligation of the left anterior descending coronary artery (LAD) as described. Reperfusion was then allowed for 24 hours or 14 days and followed by invasive monitoring of left ventricular function with a Millar tip catheter. The catheter was advanced through the left carotid artery and aorta into the left ventricle (LV) under continuous monitoring of the pressure curves until the diastolic pressure indicated localization in the LV. N-acetylcysteine (NAC, 15 mmole/animal) was administered intraperitoneally 15 minutes before the onset of ischemia, and cyclosporin A (CsA, 10 mg/kg intravenously via tail vein) was administered at the onset of reperfusion.

**Infarct Size, TUNEL Staining, Ultrastructural Analysis**

Infarct size was determined by Evans blue exclusion under conditions of LAD reoxygenation. After subsequent heart excision, 3 short axis slices were incubated with triphenyl-tetrazolium-chloride (TTC) for 10 minutes. Microscopic pictures were taken and analyzed using planimetry (ImageJ; NIH, Bethesda, MD). TUNEL-positive cells were obtained from the ischemic region using ApopTag Kit (Millipore, Schwalbach, Germany) according to the manufacturer’s guidelines.

For ultrastructural analysis, hearts were isolated and immediately fixed in 2.5% glutaraldehyde-2% parafomaldehyde (PFA). Samples were embedded in propylenoxyd and Polyemebd 812 (Plano, Wetzlar, Germany). Ultrathin sections (60 nm) were stained with uranyl acetate and lead acetate and then examined on a Zeiss (Oberkochen, Germany) 902 electron microscope at 80 kV.

**Embryonic Endothelial Progenitor Cells (eEPC) and Embryonic Stem (ES)**

**Cell-Derived Cardiomyocytes**

Vascular progenitor cells were derived from ubiquitous Txnrd2-/- murine embryos (E7.75) according to a protocol established by Hatzopoulos et al. ES cell lines were established from E3.5 embryos from matings with Txnrd2flox/flox and Txnrd2flox/flox mice. ES cell clones were stably transfected with the plasmid pCAG-3SIP-MerCreMer by electroporation and selected by 1 µg/mL puromycin as described. Subsequently, MerCreMer-expressing ES cell clones were transfected with the plasmid pMHC-neo-PGK-Hyg (a kind gift from Loren Field, Indianapolis, IN). Cardiomyocyte selection was performed by hygromycin addition at di4 after leukemia inhibiting factor (LIF) withdrawal (initiating differentiation). Activation of Cre-recombinase and excision of the floxed Txnrd2 allele was performed by 4-hydroxytamoxifen addition for 72 hours (1 µmol/L). Hypoxia (<1% oxygen) was induced by superfusing cell cultures with nitrogen for 18 hours before 4 hours’ reoxygenation in the case of cardiomyocytes, and for 6 hours before 2 hours’ reoxygenation with eEPCs. For fluorescence microscopy, DAPI (Vector Laboratories, Burlingame, CA) was used; for nuclear staining, troponin-I and α-MHC antibodies (both Santa Cruz) were used.

**Adult HL-1 Cell Knockdown of Thioredoxin Reductase-2**

To generate small hairpin RNA against murine Txnrd2, we used 5'-TACCGGGAATACCTCAAAAATGTCTGTTGACGATCTAACATTGAGAATTCTCCTCCCTTTTTTCGATATTGTGTGTTTGGG-3' (targeting 1187–1207bp in the Txnrd2 mRNA) and the scrambled sequence oligonucleotide 5'-GA TCCGGAAATGCTCTGTTGAGCTCTCCTCGTACAG-AAGCTT GAACGAGACATTTCCTTTTGTTG3'. Oligonucleotides were ligated into the pGreenPuro vector (System Biosciences). HL-1 cells were plated on fibronectin-coated plates and cultured with Claycomb media (Sigma) for transduction with lentivirus using a MOI of 1000/cell in the presence of 8 µg/mL Polybre (Sigma). Puromycin (50 µg/mL) was added 48 hours after infection for another 48 hours. Hypoxia was induced for 12 hours (<1% O2) followed by reoxygenation (1 hour).

**Protein Isolation, Protein Concentration Determination, and Immunoblotting**

Protein isolation, protein concentration determination, and immunoblotting are described in detail in the online-only Data Supplement Methods.
Determination of Reduced Glutathione

Intracellular GSH levels were measured in Txnrd2<sup>−/−</sup> cells by high-performance liquid chromatography in normoxic cells and after hypoxia/reoxygenation, as described.30

Mitochondria: Isolation and Functional Analysis

Detailed methods are described in the online-only Data Supplement Methods. In brief, mitochondria were freshly isolated from Txnrd2<sup>−/−</sup>-ic and control hearts by differential centrifugation according to standard protocols.31 Functional integrity of isolated mitochondria was routinely assessed with a Clark type oxygen electrode (Oxygraph<sup>TM</sup>, Hansatech Instruments Ltd, UK). Permeability-transition–induced swelling of mitochondrial suspensions was measured by light scattering at 540 nm, as described recently.31 The presence of noncardiomyocyte cells like vascular cells and fibroblasts, which also express Txnrd1, on I/R, aconitase activities (n=10 Txnrd1<sup>+/−</sup>-ic, n=12 Txnrd2<sup>−/−</sup>-ic, n=4 Txnrd1<sup>+/−</sup>-ic, n=4 Txnrd1<sup>−/−</sup>-ic, where n is the number of measurements; Cayman Chemicals, Ann Arbor, MI) were normalized to their normoxic counterparts (set to 100%), averaged, and subjected to statistical analyses.

Statistical Analysis

The results are given as mean±SEM. Statistical analysis of results among ≥2 experimental groups was performed with one-way analysis of variance (ANOVA). Whenever a significant effect was obtained with ANOVA, we performed multiple comparison tests among the groups using the Student Newman Keul’s procedure. For comparing just two experimental groups, Student’s t test was applied. All procedures were performed with an SPSS statistical program (Version 19) after Shapiro-Wilk and Levene testing for normality and equality of variances. Differences between groups were considered significant at P<0.05.

Results

Role of Thioredoxin Reductase-1 Deficiency During Ischemia/Reperfusion

We first investigated the impact of Txnrd1-knockout on cardiac injury inflicted by ischemia (90 minutes) and reperfusion (24 hours). Immunoblotting of heart-specific Txnrd1 knockout mice (Txnrd1<sup>−/−</sup>) (Figure 1A) revealed a steep decrease of Txnrd1 in heart tissue (Figure 1B). Low Txnrd1 quantities were still detectable due to either mosaic expression of Cre in the cardiomyocytes or, most likely, the presence of noncardiomyocyte cells like vascular cells and fibroblasts, which also express Txnrd1. On I/R, Txnrd1<sup>−/−</sup>-hearts differed from sham-operated experimental groups but did not notably differ from Txnrd1<sup>+/−</sup>-hearts in their ability to maintain a residual LV function (LVDP after I/R: 82±4 versus Txnrd1<sup>+/−</sup> 81±2 mm Hg at rest and 123±6 versus 114±4 mm Hg at 50 ng norepinephrine stimulation; no significant difference for each condition) (Figure 1C) (online-only Data Supplement Methods). Likewise, inducible Txnrd1<sup>−/−</sup>-hearts, generated by cardiomyotropic rAAV2.9-Cre transduction of Txnrd1<sup>lox/lox</sup> mice (termed Txnrd1<sup>−/−</sup>-icV) (online-only Data Supplement Figure 1A) or wild-type C57BL/6 hearts did not alter postischemic hemodynamic function (online-only Data Supplement Figure IB).

Moreover, no statistically significant difference in the postischemic prevalence of TUNEL-positive cardiomyocytes was detectable between Txnrd1<sup>+/−</sup>- and Txnrd1<sup>−/−</sup>-mice (Figure 1D), although both levels significantly exceeded normoxic (sham) levels. Consistently, infarct size was not significantly different between Txnrd1<sup>+/−</sup> and Txnrd1<sup>−/−</sup>-icV

Figure 1. Relevance of endogenous thioredoxin reductase-1 (Txnrd1) for postischemic reperfusion injury. A, Cardiospecific deletion of Txnrd1, achieved by crossing of mice carrying a floxed (►)Txnrd1 allele with a MLC2aWT/Cre mouse. B, Cardiac tissue-specific deletion was confirmed by immunoblotting. C, A Millar tip catheter placed in the left ventricle revealed no difference in left ventricular developed pressure (LVDP) of Txnrd1<sup>−/−</sup>- mice, Txnrd1<sup>+/−</sup>-icV mice (Txnrd1<sup>lox/lox</sup> induced by an AAV Cre) (online-only Data Supplement Methods), and Txnrd1<sup>+/−</sup>- mice at rest or after stimulation with intravenous norepinephrine (NE; bolus of 10–50 ng) (n=7 per group). §P<0.05 versus all ischemic/reperfusion (I/R) groups. D, TUNEL-positive cells in the ischemic myocardium were detected at a similar density in control and Txnrd1<sup>−/−</sup>-mice after I/R (n=12; low power fields of 4 hearts per group). #P<0.05 versus Txnrd1<sup>−/−</sup>-sham.
hearts (online-only Data Supplement Figure IIA and IIB). These findings indicate that endogenous Txnrd1 does not fulfill an essential function in cardioprotection against oxidative stress as provided by postischemic reperfusion following 90 minutes’ LAD occlusion.

Role of Thioredoxin-2 Overexpression During Ischemia/Reperfusion

Next, we assessed postischemic cardioprotection in murine hearts overexpressing human Txn2 (TGhTxn2).18 Under basal conditions, no significant difference was obtained for LVDP in TGhTxn2 mice subjected to I/R compared with littermate controls, although under stimulation with 25 and 50 ng of norepinephrine (NE), cardiac performance increased in TGhTxn2 mice (LVDP 134±6/110±6 mm Hg hearts at 50 ng of NE) (online-only Data Supplement Figure IIIA). Similar results were obtained for contraction velocity dP/dtmax (online-only Data Supplement Figure IIIB). These moderate effects may imply that, rather than the ROS scavenger Txn2, the corresponding reductase Txnrd2 might be of rate-limiting relevance during an I/R episode, prompting us to investigate cardiospecific Txnrd2-deficient hearts in this scenario.

Role of Thioredoxin Reductase-2 Deficiency During Ischemia/Reperfusion

We recently reported that ubiquitous inactivation and heart-specific disruption of Txnrd2 resulted in an embryonic and early postnatal lethal phenotype, respectively.20 In order to study I/R injury in mice lacking mitochondrial Txnrd2, we generated a mouse strain that lost Txnrd2 expression in cardiomyocytes on tamoxifen induction (Figure 2A and online-only Data Supplement Methods). An organ-specific decrease of Txnrd2 protein levels was evident in the heart, but not in the liver, using the C-terminal antibody 1C4 (Txnrd2−/−ic=inducible cardiac Txnrd2−/−). C. Millar tip catheter analysis revealed a significant difference in left ventricular developed pressure (LVDP) at rest and with norepinephrine (NE) stimulation at 24 hours. Contraction velocity dP/dtmax confirmed the systolic impairment (D), whereas the relaxation velocity dP/dtmin was unchanged (E). F. For left ventricular enddiastolic pressure, no difference was found among experimental groups \( P=0.14, 0.47, 0.42, \) and 0.27 at rest and with 10, 25, and 50 ng of NE. Txnrd2−/−sham n=4, Txnrd2−/−ic sham n=5, Txnrd2+/− I/R, and Txnrd2−/−ic I/R n=7. §P<0.05 versus Txnrd2−/− I/R and Txnrd2−/−ic I/R. *P<0.05 versus all other groups.
Figure 3. Txrd2-/-ic aggravates cellular detriment of posts ischemic reperfusion injury. A, Electron beam microscopy examples of sham and ischemia/reperfusion (I/R) - (60/30 minutes) treated hearts. B: Quantification revealed that mitochondrial integrity (online-only Data
124±5 mm Hg), whereas the function at rest had recovered at this time point (online-only Data Supplement Figure IVB). The deterioration of systolic function in Txnrd2-/-ic mice at 24 hours was mirrored in decay of contraction velocity dP/dtmin, (6424±521 versus 8594±363 mm Hg/s at rest, 13803±1768 versus 18897±1532 mm Hg/s with 50 ng of NE) (Figure 2D). Of note, diastolic function, assessed by relaxation velocity dP/dtmin, was not significantly altered by cardiospecific loss of Txnrd2 (−6574±457 versus −7519±1645 mm Hg/s in controls, 12012±1645 mm Hg/s in sham at rest) (Figure 2E). Moreover, no significant alterations were observed for both aconitase activity to a larger extent in Txnrd2−/− heart mitochondria with the addition of ADP. These data indicate that, with metabolic disturbances such as I/R, mitochondria with the addition of ADP. These data indicate that, with metabolic disturbances such as I/R, mitochondria with the addition of ADP. These data indicate that, with metabolic disturbances such as I/R, mitochondria with the addition of ADP. These data indicate that, with metabolic disturbances such as I/R, mitochondria with the addition of ADP. These data indicate that, with metabolic disturbances such as I/R, mitochondria with the addition of ADP. These data indicate that, with metabolic disturbances such as I/R, mitochondria with the addition of ADP. These data indicate that, with metabolic disturbances such as I/R, mitochondria with the addition of ADP. These data indicate that, with metabolic disturbances such as I/R, mitochondria with the addition of ADP. These data indicate that, with metabolic disturbances such as I/R, mitochondria with the addition of ADP. These data indicate that, with metabolic disturbances such as I/R, mitochondria with the addition of ADP. These data indicate that, with metabolic disturbances such as I/R, mitochondria with the addition of ADP. These data indicate that, with metabolic disturbances such as I/R, mitochondria with the addition of ADP. These data indicate that, with metabolic disturbances such as I/R, mitochondria with the addition of ADP. These data indicate that, with metabolic disturbances such as I/R, mitochondria with the addition of ADP. These data indicate that, with metabolic disturbances such as I/R, mitochondria with the addition of ADP.

Augmented Mitochondrial Impairment, Increased Cell Death, and Larger Infarct Size on Ischemia/Reperfusion in Thioredoxin Reductase-2 Deficient Hearts

At a cellular level, transmission electron microscopy revealed losses of regularity of mitochondrial striation and of membrane integrity as early as 60 minutes after I/R in Txnrd2-/-ic mice (Figure 3A), which exceeded the levels seen in Txnrd2+/+ hearts. A mitochondrial score consisting of swelling, loss of striation, and membrane disintegration (online-only Data Supplement Data Figure V) confirmed this observation: although no significant difference in mitochondrial integrity was observed between normal and I/R hearts, mitochondrial disintegration was obvious after I/R (Figure 3B). As assessed by the release of H2O2, heart mitochondria produced more ROS on the addition of the respiratory chain complex I- and complex II-linked substrates in the absence of rotenone (succinate and succinate+glutamate+malate group, respectively) (Figure 3C). Such reverse electron flow conditions (RET) produce ROS at complex I, and similar values were obtained for isolated heart mitochondria compared with recently reported values for skeletal muscle mitochondria. Importantly, under these conditions, Txnrd2-/-ic heart mitochondria produced a significantly higher emergence of ROS compared with Txnrd2+/+ heart mitochondria (Figure 3C). Rotenone, an inhibitor of complex I blocking the RET, led to a lower amount of H2O2 for both Txnrd2+/+ and Txnrd2-/-ic heart mitochondria, being still higher for the latter. No significant difference was observed in H2O2 levels between the Txnrd2+/+ and Txnrd2-/-ic heart mitochondria with the addition of amitocin. Interestingly, comparably low amounts of H2O2 were observed for Txnrd2+/+ and Txnrd2-/-ic heart mitochondria with the addition of ADP. These data indicate that, with metabolic disturbances such as I/R, Txnrd2-/-ic heart mitochondria may produce more ROS. This prediction was further investigated by assessing activity of the redox-sensitive mitochondrial enzyme aconitase. In fact, we detected a significant decrease of aconitase activity in mitochondria derived from whole Txnrd2-/-ic hearts subjected to I (60 minutes) and R (30 minutes) compared with myocardial Txnrd2+/+ mitochondria after I/R (Figure 3D). No difference in aconitase activity was seen under normoxic conditions (online-only Data Supplement Figure VE). In addition, the activity of aconitase was not significantly different between Txnrd1+-/ and Txnrd1-/-ic hearts after I/R (Figure 3D).

The impaired function and integrity in Txnrd2-/-ic heart mitochondria on I/R was associated with an increase in TUNEL-positive heart cells at 24 hours of reperfusion but not at rest (Figure 3E and 3F). Infarct size, a second hallmark of I/R injury, was enlarged in Txnrd2+/+ mice compared with Txnrd2+/+ controls in the acute phase 24 hours after the insult (Figure 3G and 3H) and 14 days after the insult, a time point when scar formation is complete (online-only Data Supplement Figure 4C and 4D).

Thioredoxin Reductase-2 Deficiency Sensitizes Toward Mitochondrial Pore Opening and Subsequent Cell Death

A well-documented mechanism of I/R injury is opening of mitochondrial mPTPs, which can be inhibited by the interaction of cyclosporin A (CsA) with the prolylisomerase cyclophilin D located in the mitochondrial matrix. Notably, CsA application abolished the loss of systolic function after I/R in the Txnrd2-/-ic hearts but not in the Txnrd2+/+ hearts (Figure 4A and 4B). Moreover, CsA was also able to decrease the number of TUNEL-positive cells in Txnrd2-/-ic hearts (Figure 4C) at the level of Txnrd2+/+ hearts. As delineated in Figure 4D, swelling of isolated mitochondria, a hallmark of mPTP-induced detriment, was also inhibited by CsA.

N-Acetylcysteine Rescues Postischemic Thioredoxin Reductase-2-Deficient Hearts

In order to test the hypothesis that the antioxidant NAC might compensate for the impaired regeneration of oxidized mitochondrial thiols, we first employed cell culture assays. Because murine embryonic fibroblast cell cultures from Txnrd2-/- mice did not display a detectable difference in cell death on hypoxia and reoxygenation (data not shown), we developed 3 alternative cell culture models of Txnrd2 deficiency: (1) vascular progenitor cells derived from ubiquitous Txnrd2-/ mice (eEPCs) (Figure 5A through 5C); (2) Txnrd2-deleted cardiomyocytes derived from Txnrd2flox/floxERCreER/ES cells (Figure 5D through 5F); and (3) an adult murine cardio-
myocyte cell line (HL-1) in which Tnxrd2 expression was experimentally decreased (Figure 5G and 5H). We analyzed the reduced glutathione pool after hypoxia/reoxygenation of eEPCs because glutathione (GSH) has been reported to be a valid surrogate parameter for thiol supply in various model organisms. As depicted in Figure 5B, NAC increased the GSH content in Txnrd2-/eEPCs to levels found in untreated wild-type cells. Pretreatment with NAC caused a dramatic increase in cell survival of Txnrd2-deficient eEPCs after hypoxia and reoxygenation (Figure 5C) similar to pretreatment with the antioxidants GSH or tocopherol.

Analysis of ES cell-derived, contracting Txnrd2flox/floxMerCreMer cardiomyocytes (online-only Data Supplement Video I) was performed after 72 hours’ induction with tamoxifen when deletion of Tnxrd2 expression was obtained (Figure 5D). Txnrd2-/ES cell-derived cardiomyocytes staining positive for the cardiomyocyte-specific markers MHC and actinin (Figure 5E) displayed an increased vulnerability toward hypoxia-reoxygenation stress, which was reversed to Txnrd2+/levels by NAC application (Figure 5F, online-only Data Supplement Video II). A similar effect was obtained in HL-1 cardiomyocytes, displaying a reduced Tnxrd2 expression after lentiviral transfection of Tnxrd2-shRNA (Figure 5G). Again, decreased Tnxrd2 expression sensitized toward hypoxia-reoxygenation induced cell death unless NAC was supplied (Figure 5H).

To verify that NAC is capable of normalizing the phenotype of Tnxrd2-/ic in vivo, we applied NAC intraperitoneally 30 minutes before the onset of ischemia. In fact, performance reached the functional levels of Tnxrd2+/ mice treated with NAC (Figure 6A and 6B). NAC application even abolished the excess cell death (Figure 6C) and the mitochondrial detriment (Figure 6D and 6E) observed in postischemic Txnrd2-deficient hearts. Moreover, NAC was capable of reducing butyl-hydroperoxide–induced mitochondrial ROS burden to a large extent (Figure 6F).

**Discussion**

Recently, mutations in the mitochondrial Tnxrd gene have been identified to cause dilated cardiomyopathy. In the present study, we provide evidence for an essential role of endogenous mitochondrial Tnxrd2 in controlling I/R injury of the heart. To this end, we used mice in which exons 15–18 of the remaining Tnxrd2 allele were flanked by Lox-P sites. On induction with tamoxifen, a cardiospecific Cre-recombinase translocated to nuclei and excised the remaining Tnxrd2 allele. This strategy resulted in an approximately 70% loss of Tnxrd2 expression in cardiac cells in vivo. Such hearts subjected to LAD occlusion (90 minutes) and subsequent reperfusion displayed an early increase of mitochondrial swelling (Figure 3A and 3B), followed by enhanced cardiomyocyte cell death (Figure 3C and 3D) and an aggravated I/R-induced functional detriment (Figure 2C through 2F). An increased infarct size was found in Tnxrd2-deficient hearts after 24 hours (Figure 3G and 3H) and up to 14 days of reperfusion (online-only Data Supplement Figure 4C). Moreover, the sensitivity of Tnxrd2-deficient cells for hypoxia/reoxygenation-induced detriment was blunted by exog-
responsive application of the antioxidants glutathione, α-tocopherol, and NAC, the latter replenishing the reduced glutathione pool (Figure 5B and 5C). After peritoneal application, NAC was capable of abolishing not only the postischemic increase in the rate of myocardial cell death, but also the loss of cardiac function of Txnrd2-deficient mice (Figure 6A through 6D). Because the cysteine provider NAC is known to attenuate cellular vulnerability caused by a lack of thiol-based antioxidant systems, be it either glutathione41 or thioredoxin reductase-2,20 this NAC effect indicates that Txnrd2 deficiency significantly accentuates oxidative stress. Apparently, this phenomenon is not overcome by Txn2 upregulation in Txnrd2-deficient mice (Figure 2B), most likely due to the inability of mitochondria to regenerate oxidized Txn2 in the absence of Txnrd2.

Programmed cardiomyocyte death has evolved as a hallmark of cellular detriment during reperfusion,42 featuring direct caspase activation by cytochrome C released from...
Bax/Bak-permeabilized mitochondria. Moreover, postischemic reperfusion is sufficient to induce opening of the mitochondrial permeability transition pore (mPTP). ROS are known to contribute to the opening of the mPTP, triggering mitochondrial membrane potential (ΔΨm) breakdown, accumulation of fluids in the matrix, swelling and rupture of the mitochondrial outer membrane, and subsequent cell death. CsA, which inhibits cyclophilin D activity and subsequent mPTP opening, improved I/R injury in patients. In our models, CsA was able to reverse the increased sensitivity of Txnrd2-deficient hearts and cells toward mPTP opening to background levels (Figure 4A through 4D).

Because thiol supplementation via NAC or inhibition of mPTP opening by CsA prevents excessive I/R injury, it is reasonable to conclude that lack of Txnrd2 expression specifically impairs detoxification of ROS generated within mitochondria. In this context, it is noteworthy that cardiomyocyte-specific Txnrd1 deficiency did not affect the postischemic outcome (Figure 1). However, in a different model of ischemia (30 minutes), application of recombinant human Txn1 helped to reduce infarct size and apoptotic index. It appears likely that with increasing depth of I/R injury, mitochondrial Txnrd becomes more prominent as Txn regenerating enzyme. Supporting this notion, NAC as an exogenous thiol-providing ROS scavenger did not alter the functional state of postischemic Txnrd2+/− control mice in our model. Consistently, in a recent clinical study, NAC (2 x 1200 mg/d) did not increase the myocardial salvage of patients with ST elevation myocardial infarction, bona fide Txnrd2 carriers. Only in the instance of Txnrd2 deficiency did thiol supplementation by NAC attenuate I/R injury, enabling a functional improvement to the level of Txnrd2+/− control mice (Figure 6A and 6B).

In summary, we were able to demonstrate for the first time that cardiospecific deletion of Txnrd2 in the adult mouse heart induces an excessive vulnerability to I/R-induced injury. The underlying cellular mechanisms were increased mitochondrial ROS generation, impaired glutathione levels, increased sensitivity to mPTP opening, mitochondrial swelling, and, consequently, increased cell death and enhanced functional detriment. Rescue from Txnrd2 deficiency-induced cardiomyocyte injury, which was provided by N-acetylcysteine as well as CsA, points to the ability of Txnrd2 to block ROS-mediated mPTP opening in the advent of I and R of the heart. Taken together, our results highlight the role of the mitochondrial thioredoxin system in cardiomyocyte I/R injury.

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Disclosures

None.

References


reperfusion injury, although a phenotype of dilated cardiomyopathy has been associated with mutations in Txnrd2. If functional impairment of Txnrd2, eg, by mutation, is clinically associated with increased myocardial ischemia–opening are relevant mechanisms provided by endogenous mitochondrial thioredoxin reductase. Currently, it is unknown if functional impairment of Txnrd2, eg, by mutation, is clinically associated with increased myocardial ischemia–reperfusion injury, although a phenotype of dilated cardiomyopathy has been associated with mutations in Txnrd2.

**CLINICAL PERSPECTIVE**

Although clinical data on successful treatment of myocardial ischemia and reperfusion injury are scarce, the role of reactive oxygen species (ROS) caused by an imbalance of oxygen supply and proper mitochondrial utilization is well established. Cellular defense mechanisms against the reperfusion-incited wave of ROS, such as oxidation of reduced glutathione and thioredoxin, depend on regenerating enzymes in order to be available during the whole period of enhanced ROS formation. In the current experimental study, we provide genetic evidence that deletion of mitochondrial thioredoxin reductase (Txnrd2) in an adult mouse results in increased cardiomyocyte vulnerability during early postischemic reperfusion. In vivo, Txnrd2 deletion caused increased infarct size and cardiomyocyte apoptosis, whereas functional recovery of postischemic hearts was significantly impaired by the absence of Txnrd2. Of note, none of these hallmarks of reperfusion was obtained in mice lacking cytosolic thioredoxin reductase (Txnrd1). The observation that the mitochondrial permeability transition pore inhibitor cyclosporin A and the cysteine-rich antioxidant N-acetyl-cysteine were able to rescue the Txnrd2 phenotype indicates that indeed ROS scavenging and direct or indirect prevention of mitochondrial permeability transition pore opening are relevant mechanisms provided by endogenous mitochondrial thioredoxin reductase. Currently, it is unknown how functional impairment of Txnrd2, eg, by mutation, is clinically associated with increased myocardial ischemia–reperfusion injury, although a phenotype of dilated cardiomyopathy has been associated with mutations in Txnrd2.
Mitochondrial Thioredoxin Reductase Is Essential for Early Postischemic Myocardial Protection


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Supplemental Methods:

Heart-specific disruption of Txnrd1 and Txnrd2

All animal experiments were approved by the Bavarian Animal Care and Use Committee and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996, Approved Institution #A5637-01). We used male mice at age 8-12 weeks. For Txnrd1⁻/⁻, no difference in heart weight and body weight was found compared to background and heterozygous mice. For Txnrd2, tamoxifen induction reduced heart weight and weight gain of the mice, which, however, was identical for induced Txnrd2-deletion and control groups.

Heart-specific inactivation of Txnrd1 was achieved by using the MLC2a-Cre transgenic mouse line as described. Mice overexpressing human thioredoxin-2 (TghTrx2) were created by Dr. Jiyang Cai in collaboration with Dr. Dean P. Jones, Emory University, Atlanta, Georgia. Cardiac tissue specific disruption of Txnrd2 was achieved by crossing Txnrd2floxt/⁻ mice with a heart specific tamoxifen inducible Cre recombinase expressing strain and feeding for four weeks with chow containing tamoxifen citrate (= Txnrd2⁻/-ic). The latter protocol was also applied to control (heterozygote mice) and sham groups to be compared to Txnrd2⁻/-ic mice.

Prior to the ischemia / reperfusion experiments, the tamoxifen-inducible Txnrd2 knockout mice were maintained at least for four weeks on a standard diet (Altromin, Lage, Germany) in order to exclude possible side effects of tamoxifen.

Ischemia and reperfusion

Ischemia of the heart was induced for 90 min by ligation of the LAD as described. Reperfusion was then allowed for 24h or 14d and followed by invasive monitoring of left ventricular function with a Millar tip catheter. The Catheter was advanced through the left
carotid artery and aorta into the left ventricle (LV) under continuous monitoring of the pressure curves, until the diastolic pressure indicated localization in the left ventricle. N-acetylcysteine (NAC, 15mmoles/animal) was administered intraperitoneally 15 min before the onset of ischemia, cyclosporin A (CsA, 10mg/kg i.v. via tail vein) at the onset of reperfusion.

**Determination of infarct area**

After 24h, mice were intubated and Evans blue 2% solution (Sigma-Aldrich, Seelze, Munich, Germany) was injected into the LV after LAD reocclusion to stain the non-ischemic area. The heart was then explanted, placed in optimal cutting temperature compound (OCT, Sakura, Tokyo, Japan) and frozen at -20°C for 30 minutes. Thereafter, the hearts were cut into 3 slices, beginning at the LAD suture. The slices were placed for 10min into triphenyltetrazolium chloride 1,5% solution (TTC, Sigma) at 37°C and microscope pictures of the stained slice were analysed using planimetry (ImageJ, NIH, Bethesda, USA).

**TUNEL staining**

For detection of apoptotic cells, sections of infarcted hearts were stained using ApopTag® Kit (Millipore, Schwalbach, Germany), according to the manufacturer’s guidelines. Nuklei were counterstained with DAPI (Vectashield®, Vector Laboratories, Burlingame, CA, USA). TUNEL positive cells were obtained from the ischemic region (10 randomly selected high power fields per heart, 4 hearts per group) and related to total number of DAPI positive cells in percent. Pictures were analysed by 2 independent investigators in a blinded fashion.

**Ultrastructural analysis**

Hearts were isolated and immediately fixed in 2.5% glutaraldehyde-2% paraformaldehyde (PFA) in 0.1 M sodium cacodylate buffer (pH 7.2) for 24h, rinsed three times in the same buffer, and postfixed in 1% osmium tetroxide-1.5% potassium ferrocyanide in potassium cacodylate buffer for 2h. Samples were washed three times, dehydrated, and embedded in Propylenoxyd and Polyembed 812 (Plano, Wetzlar, Germany). Ultrathin sections (60nm)
were stained with uranyl acetate and lead acetate and then examined on a Zeiss 902 electron microscope at 80 kV.

Embryonic endothelial progenitor cells (eEPC) and ES cell derived cardiomyocytes

Vascular progenitor cells were derived from ubiquitous \textit{Txnrd2}/- murine embryos (E7.75) according to a protocol established by A.K. Hatzopoulos \textsuperscript{6}.

Embryonic stem cell lines were established from E3.5 embryos from matings of \textit{Txnrd2}+/flax with \textit{Txnrd2}flax/flax mice \textsuperscript{7}. The cell line was kept in media containing DMEM, 15% FCS, 1% glutamine, 50 U/ml penicillin G, 50 µg/ml streptomycin 50µM and β–Mercaptoethanol. The cell clones were grown in high glucose Dulbecco’s modified Eagle medium supplemented with 10% heat-inactivated ES qualified fetal calf serum, 2mM L-glutamine, 50 U/ml penicillin, 50µg/ml streptomycin, 1x nonessential amino acids, 0.4mg/ml GENETICIN (G418) (all reagents GibCO BRL, Germany), and 0.1mM β-mercaptoethanol (Sigma, Germany). They were kept undifferentiated under feeder free conditions by addition of 1000 units/ml purified recombinant mouse leukemia inhibitory factor (ESGRO; Life Technologies, Inc., Grand Island, N.Y.). Cells were maintained at 37°C in a humidified atmosphere of 5% CO2/95% air. Monolayers were passaged by trypsinization at confluence of 70–80%. In vitro differentiation was initiated as follows: ES cells were harvested with 0.25% trypsin-EDTA and dissociated cells were transferred to bacteriological dishes at a density of 2 x 10^5 ES cells/ml in ISCOVE’s modified Eagle medium (Sigma) supplemented with 10% heat inactivated fetal calf serum, 2mM L-glutamine, 50U/ml penicillin, 50µg/ml streptomycin, 1x nonessential amino acids (all reagents Life Technologies, Inc.), and 450µM betamonothioglycerol (Sigma). After 2 days, EBs were transferred to new medium. At day 6, EBs with a similar size were plated onto gelatin-coated tissue culture dishes. The growth medium for the attached differentiation cultures was changed every day. Antibiotic cardiomyocyte selection was performed by addition of hygromycin at d14 after the induction of differentiation.
After appearance of beating cardiomyocytes, activation of Cre-recombinase and excision of the floxed \textit{Txnrd2} allele was performed by adding 1µM tamoxifen for 72h. Hypoxia (<1% oxygen) was induced by superfusing cell cultures with nitrogen for 18h before reoxygenation (4h) in the case of cardiomyocytes, and for 6h before 2h reoxygenation with eEPCs. For fluorescence microscopy, troponin-I and -MHC antibodies (all Santa Cruz) as well as DAPI (Vector Laboratories, Burlingame, CA USA, for nuclear staining) were used.

\textit{Adult HL-1 cell knockdown of thioredoxin reductase-2}

To yield small hairpin RNA against murine \textit{Txnrd2}, we used 5’ATCCGGAAATCCTCAACCTTAATGCTTCTGTCCAGACATTAAGGTGGAGATT TCCCTTTTTG -3’ (targeting 1187-1207bp in the \textit{Txnrd2} mRNA) and the scrambled sequence oligonucleotide 5`GATCCGGAATGTCTCGTTCAGAGCTCTTCCTGTCAGAGCTCTGAACGAGACATTTCCTTTTTG3’. Oligonucleotides were ligated into the pGreenPuro vector (System Biosciences, USA). HL-1 cells were plated on fibronectin coated layers and cultured with Claycomb media (Sigma) for transduction with lentivirus using a MOI of 1000/cell in the presence of 8µg/ml Polybren (Sigma). Puromycin (50µg/ml) was added 48h after infection for another 48h. Hypoxia was applied for 12h (<1% O2) followed by reoxygenation (1h).

\textit{Protein isolation, protein concentration determination, and immunoblotting}

Cells and tissues were lysed in LCW lysis buffer (0.5% TritonX 100, 0.5% Sodium Deoxycholate Salt, 150 mM NaCl, 20 mM Tris, 10 mM EDTA, 30 mM Na Pyrophosphate, pH 7.5) in the presence of a protease inhibitor cocktail (Roche, Mannheim, Germany), briefly sonicated. Tissue samples were additionally minced by using an overhead stirrer (Eurostar RW16, IKA-Labortechnik, Staufen, Germany). Protein concentration was determined by the DC Protein Assay (Bio-Rad, München, Germany) according to manufacturers’ instructions. Western Blotting was performed using following primary antibodies: anti-Txn1 (1:2000; Cell Signaling Technology, New England Biolabs, Frankfurt am Main, Germany), anti-Tnrxrd1
(1:2000; Upstate, Millipore, Schwalbach, Germany), anti-Txn2 (1:1000; R&D Systems, Wiesbaden-Nordenstadt, Germany), anti-Txnr2d (1:500; Santa Cruz Biotechnology, Heidelberg, Germany) and Txnr1- and Txnr2-specific antibodies (clone# 1E12 and clone# 1C4, respectively) as described (Mandal et al, 2010); anti-alpha-Tubulin (1:2000; Sigma-Aldrich, Deisenhofen, Germany) served as control. Detection was achieved by incubation with HRP-conjugated secondary antibody and visualized by ECL detection (GE Healthcare, München, Germany) on a hyperfilm.

**Determination of reduced glutathione**

Intracellular GSH levels were measured in Txnr2d−/− cells by high performance liquid chromatography (HPLC) in normoxic cells and after hypoxia / reoxygenation (H/R), as described 8. In brief, cells were exposed to N-ethylmaleimide (NEM) solution in perchloric acid and GSH determined as its NEM.adduct. This extraction technique instantly stops all metabolism of glutathione.

**Mitochondria isolation**

Mitochondria were isolated by differential centrifugation according to standard protocols as previously described 9. Briefly, freshly removed mouse hearts were minced using a razor blade and homogenized in ice-cold isolation buffer (IB: 5mM TES, 0.3M sucrose, 0.2mM EGTA, 0.1%BSA, pH7.2 with KOH) using 5 strokes in a tight fit glass/glass homogenizer. Homogenates were cleared from debris and nuclei by centrifugation at 1400g (10min at 4°C), and mitochondria were pelleted at 9,000 g (10min at 4°C). Mitochondria were washed once at 9,000g (10min, 4°C) and resuspended in IB. Protein concentrations were determined by the Bradford assay. Functional integrity of isolated mitochondria was routinely assessed by standard respiratory measurements with a Clark type oxygen electrode (Oxygraph™, Hansatech Instruments Ltd, UK).

**Mitochondrial swelling assay**
Permeability-transition induced swelling of freshly isolated mouse heart mitochondria suspensions was routinely measured by light scattering at 540nm in a micro-plate absorbance reader (µ-Quant™, Bio-Tek, Bad Friedrichshall, Germany) at RT, as described recently ⁹.

The final assay volume was 200µl, containing mitochondria at 0.4mg/ml in “swelling buffer” (10mM MOPS Tris, pH 7.4, 200mM sucrose, 5 mM succinate, 1 mM Pi, 10µM EGTA and 2µM rotenone). CsA (5µM) was added 5min before Ca²⁺ (100µM). PT-induced swelling of freshly isolated mouse heart mitochondria suspensions was routinely measured by light scattering at 540nm in a micro-plate absorbance reader (µ-Quant™, Bio-Tek, Bad Friedrichshall, Germany) at RT. The final assay volume was 200µl, containing mitochondria at 0.4mg/ml in “swelling buffer” (10mM MOPS Tris, pH 7.4, 200mM sucrose, 5 mM succinate, 1mM Pi, 10µM EGTA and 2µM rotenone). CsA (5µM) was added 5min before Ca²⁺ (100µM).

Mitochondrial ROS assay

The generation of ROS in isolated mitochondria, challenged by different chemicals, was essentially measured as described previously ¹⁰. Briefly, freshly isolated mitochondria were stained for 10 min at 0°C with 2mM 2’,7’-dichloro-dihydro-fluorescein-diacetate (D-399, Invitrogen GmbH, Karlsruhe, Deutschland) in IB. After a washing step, stained mitochondria were incubated in swelling buffer and challenged by addition of 500µM t-BuOOH either with or without combination of 1mM NAC. After addition of t-BuOOH, the development of radical species was monitored by the fluorescence of dichlorofluorescein with a SynergyHT2™ Multi-Mode Microplate Reader (Bio-Tek, Bad Friedrichshall, Germany) (Ex 485/20; Em 528/20) ¹⁰.

H₂O₂-Assay

Superoxide production was measured enzymatically after conversion to H₂O₂ and expressed as H₂O₂ release from intact mitochondria as described ¹¹. H₂O₂ was detected by the oxidation of nonfluorescent Amplex™ Red to fluorescent Resorufin Red. The Resorufin formation was
monitored at $\lambda_{ex}$ of 540/25nm and $\lambda_{em}$ of 620/40nm with a SynergyHT2™ Multi-Mode Microplate Reader (Bio-Tek, Bad Friedrichshall, Germany). Values were blank corrected and converted into H$_2$O$_2$ concentrations using a H$_2$O$_2$ standard curve.

**Aconitase Assay**

Mitochondria isolated from whole hearts (60 min ischemia, 30 min reperfusion) were analysed according to manufacturer’s instructions (Cayman Chemicals, Ann Arbour, USA).

**Statistical analysis**

The results are given as mean ± SEM. Statistical analysis of results between >2 experimental groups was performed with one way analysis of variance (ANOVA) Whenever a significant effect was obtained with ANOVA, we performed multiple comparison tests between the groups using the Student Newman Keul’s procedure. For comparing just two experimental groups, Student’s t-test was applied. All procedures were performed with an SPSS statistical program (Version 17.0.2.). Differences between groups were considered significant for $p<0.05$. 
Supplemental Reference List


Supplemental Table 1

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Supplemental Table 1: Although heart rate varies between sham and ischemia/reperfusion conditions, no statistical significant difference is detectable between ischemia/reperfusion groups (Txnrd2+/+ vs. Txnrd2-/-ic), with or without treatment of CsA or NAC or vs. Tgh<sup>loxP</sup> during invasive measurements.
Supplemental Figure 1 A: Robust expression of Cre 14d after AAV2.9 transfection. Pictures show confocal microscopy images of heart tissue from tomato mice 14d after transfection with 5x10^{12} virus particles AAV2.9 expressing Cre-recombinase, changing tissue fluorescence from red (tomato) to green (eGFP) (upper panels) and control tissue of untransfected tomato mice (lower panel). 

B: No difference between left ventricular developed pressure in Txnr7-/- mice compared to C57/B6-WT mice. (n=7, §=p<0.05)
Supplemental Figure 2 Role of Txnrd 1 during myocardial ischemia and reperfusion

A,B: At 24h, infarct size (white area) is similar compared to AAR (red) in Txnrd1-/-icV and Txnrd1+/+ mice (n=4 hearts per group). C,D: Comparison of mice with or without treatment of tamoxifen before hemodynamic analysis. No difference was observed in analysis of left ventricular function of C57/B6 mice either receiving regular diet or tamoxifen for 4 weeks followed by 4 weeks of regular diet (n=7, $p<0.05$ vs IR).
Supplemental Figure 3: Relevance of thioredoxin-2 overexpression for postischemic reperfusion injury

A: C57BL/6J mice with overexpression of human Txn2 under the control of the chicken β-actin promotor (TghTxn2, n=8) and littermate controls (WT, n=6) were subjected to ischemia (90min) and reperfusion (24h), or sham-operated (TghTxn2 and WT, n=5 each). Millar tip catheter analysis revealed no difference in left ventricular developed pressure (LVDP, A) or contraction velocity (dP/dt\text{max}, B) at rest. However, improved cardiac reserve was unmasked after 25 and 50ng boli of i.v. norepinephrine (NE). ¶=p<0.05 TghTxnI/R vs WT-I/R, §=p<0.05 sham vs. I/R groups.)
Supplemental Figure 4: Relevance of Txnrd2-deficiency in cardiomyocytes during ischemia-reperfusion injury

A: Additional immunoblotting of cardiac tissue of Txnrd2+/- and Txnrd2-/-ic hearts with the polyclonal Txnrd2 antibody (Sc46279), which is directed against the N-terminus, showed a clear reduction in Txnrd2 levels. No band was detected at 45kd, indicating no stable formation of a C-terminally truncated Txnrd2 protein after removal of Txnrd2 exons 15 – 18 (see the targeting strategy described in Fig. 2A). Immunoblotting against Trx1 and Txnrd1 showed that both proteins are not upregulated in response to inducible Txnrd2 deletion. (-/+ = Txnrd2+/-; -/-ic = Txnrd2-/-ic) B: Analysis of left ventricular developed pressure (LVDP) at 14 d did not detect a significant difference among Txnrd2-/-ic and Txnrd2+/+ hearts at rest, but a distinct loss of LVDP at increasing NE boli (*=p<0.05 vs. Txnrd2+/-, §=p<0.05 vs. sham Txnrd2+/-). §=p<0.05 vs. I/R groups. C,D: At d14, infarct size (blue area) was larger in Txnrd2 treated hearts than in control hearts (n=5 hearts per group), #=p<0.05 vs. Txnrd2+/-.
Supplemental Figure 5: Mitochondrial score: Quantification of mitochondrial damage.

Mitochondrial score: Quantification of mitochondrial damage. Mitochondrial structure in electron beam microscopy (EBM) pictures was assessed in context of swelling and intactness of inner and outer membrane. The pictures shown in Supplemental Fig 5A-D are randomly chosen examples to visualize and specify the development of the score. A Three points were counted if nearly all mitochondria were intact. B In the case of destruction of up to one third of mitochondria two points were assigned. C Loss of more than one third and up to two third of mitochondria was counted with one point, D destruction of more than two third of mitochondria was rated with zero points. Pictures show examples of each category. 15 EBM samples per heart, n=3 hearts of each experimental group. Fig E displays the absolute values of aconitase activities of mitochondria isolated from Txnrd2+-/- or Txnrd2-/-ic hearts at sham or ischemia / reperfusion (I/R) conditions (60min ischemia, 30min reperfusion). These values differ significantly (* Txnrd2-/-ic, sham vs I/R; # Txnrd2+-/- I/R vs. Txnrd2-/-ic I/R, p<0.05 n=4 measurements for each, Txnrd2+-/- and Txnrd2-/-ic normoxic, and n=12 and 10 measurements for Txnrd2+-/- I/R and Txnrd2-/-ic I/R, respectively, values given±sd) and reflect the impact of ischemia / reperfusion.
Supplemental Figure 6: Rescue of exacerbated ischemia and reperfusion injury of Txnrd2-/-ic hearts via Cyclosporin A (CsA)

A,B: At 24h, infarct size (white area) is slightly reduced in CsA-treated Txnrd2+- hearts, though these differences were not significant (n=4 hearts per group).
Supplemental videos 1-4: After differentiation of ES cell derived cardiomyocytes, tamoxifen was added to induce the knock-out. After 72h cells were challenged with 18h of hypoxia (H) followed by 4h reoxygenation (R). Videos show these cells before and after H/R. **SV 1 and 2**: Beating Tnrd2-/- ES cell derived cardiomyocytes before (SV1) and after H/R show significant reduction of beating frequency and strength. **SV 3 and 4**: Addition of 500µM NAC before hypoxia attenuates this posthypoxic loss of function.
미토콘드리아내 항산화효소가 초기 허혈성 심근을 구제한다면?

백 상홍 교수 가톨릭대학교 서울성모병원 순환기내과

Summary

배경

허혈-재관류(ischemic reperfusion, I/R) 후 과도하게 생성되는 ROS(reactive oxygen species)는 심근조직의 손상이나 심장의 기능적 부전에 높은 연관성을 보인다. 심근세포는 다양한 미토콘드리아를 함유하며, in vitro나 in vivo 연구에서 미토콘드리아는 ROS 생성의 주요 세포내 기관이다. I/R 손상 후 미토콘드리아는 다양한 ROS를 생성하지만, 심근세포의 내재성 ROS 억제 시스템(미토콘드리아 망간 SOD(mitochondrial manganese superoxide dismutase), 과산화 음이온 억제인자(detoxifying O₂⁻, catalase, GPx(glutathione peroxidase))에 의해 회복된다. Thioredoxin(Trx)/Thioredoxin reductase(TrxR)의 시스템은 peroxiredoxin(Prx)과 유사한 ROS의 억제 시스템인데, Trx-1과 TrxR-1은 주로 세포질에 분포하고, Trx-2와 TrxR-2는 주로 미토콘드리아에 분포하며, Trx-3 및 TrxR-3는 주로 정소에 분포한다. Trx-1은 허혈 후 생성된 ROS를 억제하여 심근세포의 세포사멸 및 경색 크기를 감소시키는 반면, Trx-2와 TrxR-2의 심장 보호효과에 대한 연구는 미미하다. Trx-2/TrxR-2 시스템은 미토콘드리아의 ROS 조절 에 핵심적인 역할을 담당하며, 미토콘드리아는 심근세포 총 부피의 약 30%를 차지하고 있으므로 심장 보호효과의 연관성이 중요할 것이다. 최근 Ang II(angiotensin II)에 대한 Trx-2의 조절이 심장 부피에 잠재적 영향성이 있음을 보고하고 있다. TrxR-1과 TrxR-2의 심장 I/R 손상의 조절 기전을 확인하기 위하여, 본 연구팀은 TrxR-1과 TrxR-2의 발현을 심장에 특이적으로 억제하는 시스템을 개발하고 이들 유전자 기능적 분석을 수행하였다.

방법 및 결과

1. I/R 중 TrxR-1 결핍의 역할

I/R 손상 후 TrxR-1의 작용을 알아보기 위하여 TrxR-1⁻/⁻과 TrxR-1⁻/⁻ 마우스를 제작한 결과, 심장 기능 및 심근세포의 세포사멸에 영향을 미치지 않았다(LVDP(left ventricular developed pressure) 및 TUNEL assay). Cardiomyotropic TrxR-1⁻/⁻ 마우스를 제작하여 I/R 후 허혈 면적을 측정한
결과, TrxR-1⁻/⁻과 대조군 사이의 허혈 조직 부분의 면적 차이가 없었다. 이러한 결과는 TrxR-1의 심장기능 보호효과가 미미한 것을 시사한다.

2. I/R 중 Trx-2 과발현의 역할
Trx-2 과발현 TG 마우스 정립 후, Trx-2 과발현 TG 마우스에서 대조군과 LVDP에 차이가 없고, ventricle contraction velocity(dP/dt\text{max})에서는 resting time의 차이가 없으나, norepinephrine 50mg 투여 후 차이가 있었다. 위의 중립적 실험 결과는 Trx-2와 TrxR-2의 효소 재한반응(rate-limiting)에 의한 결과로 생각되므로, 심장 특이적 Trx-R-2 결손 모델을 정립하였다.

3. I/R 중 TrxR-2 결핍의 역할

4. TrxR-2 결핍 심장에서 I/R 중 미토콘드리아 손상
세포사망 및 심근경색 크기의 증가

5. TrxR-2 결핍은 미토콘드리아 구명을 개방하여 세포 사망에 의함
미토콘드리아의 permeability transition pore(PTP)는 I/R 손상 후 열려 미토콘드리아의 손상을 가중시킴으로, 미토콘드리아 손상의 지표로 활용된다. PTP는 cyclosporine A(CsA)와 미토콘드리아 기질에 위치한 cyclophilin D와 상호작용하여 PTP의 열림을 억제한다. TrxR-2⁻/⁻에 I/R 후 CsA의 투여는 심장기능 손상을 회복하지만, TrxR-2⁻/⁻에서는 회복하지 못하였다. TrxR-2⁻/⁻의 I/R 후 세포사멸 심근세포 및 미토콘드리아의 손상을 감소시킨다.

6. N-Acetyl-cystein은 허혈 후 TrxR-2 결핍 심장을 구제
다양한 세포[EPC(endothelial progenitor cell), contracting cardiomyocyte, HL-1]에서 TrxR-2의 결손에 NAC가 미치는 영향을 확인하였다. EPC에서 NAC 투여 후, GSH(glutathione)의 함량이 증가하고, 다른 항산화제를 투여한 결과에서도 세포의 생존이 유의하게 증가하였다. Contracting cardiomyocyte에서는 TrxR-2의 발현을 tamoxifen 투여 후 감소하게 하였으며, 72시간째 유의하게 감소하였고, 심근 표지자인 α-MHC, actinin의 발현이 hypoxia-reoxygen(H/R) 후에 감소되었다가 NAC 투여 후 회복하였고, HL-1에서도 유사하게 NAC 투여 후 세포사멸을 확인하였다. TrxR-2⁻/⁻에 NAC를 IP(intraperitoneal)로 투여한 후 I/R를 수행한 결과는 심장의 기능(LVDP, dP/dt\text{max})이 회복됨을 확인하였다. 또한, NAC 투여는 TrxR-2⁻/⁻의 세포사멸을 감소시켰고 미토콘드리아의 손상 및 ROS의 생산을 감소시켰다.
결론
TrxR의 유전자 변형은 확장성 심근염의 발생을 유도한다고 보고되었다. 이의 연구 결과는 TrxR-2-/-가 I/R 후의 미토콘드리아의 기능적 조절을 통하여 심장기능 부전에 중요한 역할을 미칠을 증명한 연구 결과이다. TrxR-2-/-는 I/R 후 미토콘드리아의 기능적 부전을 통해 심장의 기능을 손상시켰으며, NAC 투여 후 회복을 시켰으나, TrxR-1-/-에서는 확인되지 않았다. 그러나 다른 연구들에서 Trx-1이 경색 크기에 세포사멸 index를 감소시켰다는 보고가 있으며, 임상에서 NAC 투여만으로 심장기능을 회복시키지 못하는 한계성을 보여, 이는 I/R 손상이 약화될수록 미토콘드리아의 TrxR의 중요성이 더욱 더 증가할 것으로 추정된다.

Commentary
I/R 손상 후 생성된 ROS를 제거하기 위한 방어기전이 존재하지만, 미토콘드리아의 조절 환경으로 I/R 후 심장기능의 회복에 대한 임상적인 성공 사례는 없었다. 본 연구는 ROS의 생성 조절에 미토콘드리아가 미치는 영향에 대한 규명으로 비교적 잘 진행되었고, TrxR-2 결손이 I/R 후 심근세포의 기능부전을 초래하며, 경색부위 증가 및 세포사멸을 촉진하였다. TrxR-2의 돌연변이에 의한 기능적 부전은 확장성 심근증 유발뿐만 아니라, I/R 후 심장기능의 손상을 야기하는 데도 관여할 것으로 보이지만 추가적인 연구가 필요하다.
Mitochondrial Thioredoxin Reductase Is Essential for Early Postischemic Myocardial Protection

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Background—Excessive formation of reactive oxygen species (ROS) during postischemic reperfusion significantly contributes to tissue injury and functional deterioration after myocardial ischemia/reperfusion. Especially, mitochondrial reactive oxygen species are capable of opening the mitochondrial permeability transition pore, a harmful event in cardiac ischemia/reperfusion. Thioredoxins are key players in the cardiac defense against oxidative stress. Mutations in the mitochondrial thioredoxin reductase (thioredoxin reductase-2, Txnrd2) gene have been recently identified to cause dilated cardiomyopathy in patients. Here, we investigated whether mitochondrial thioredoxin reductase is protective against myocardial ischemia/reperfusion injury.

Methods and Results—In mice, α-MHC-restricted Cre-mediated Txnrd2 deficiency, induced by tamoxifen (Txnrd2-/-ic), aggravated systolic dysfunction and cardiomyocyte cell death after ischemia (90 minutes) and reperfusion (24 hours). Txnrd2-/-ic was accompanied by a loss of mitochondrial integrity and function, which was resolved on pretreatment with the reactive oxygen species scavenger N-acetylcysteine and the mitochondrial permeability transition pore blocker cyclosporin A. Likewise, Txnrd2 deletion in embryonic endothelial precursor cells and embryonic stem cell-derived cardiomyocytes, as well as introduction of Txnrd2-shRNA into adult HL-1 cardiomyocytes, increased cell death on hypoxia and reoxygenation, unless N-acetylcysteine was coadministered.

Conclusions—We report that Txnrd2 exerts a crucial function during postischemic reperfusion via thiol regeneration. The efficacy of cyclosporin A in cardiac Txnrd2 deficiency may indicate a role for Txnrd2 in reducing mitochondrial reactive oxygen species, thereby preventing opening of the mitochondrial permeability transition pore.

Key Words: reactive oxygen species • ischemia reperfusion injury • infarct size

Excessive formation of reactive oxygen species (ROS) during postischemic reperfusion significantly contributes to tissue injury and functional deterioration of the heart. Cardiomyocytes contain a high amount of mitochondria to provide energy equivalents for generating force. However, mitochondria are the major site of generation of an oxygen radical burst on reperfusion in vitro and in vivo. Under normoxic conditions, in vitro ROS formation occurs at a rate of approximately 1% to 2% of oxygen consumption. In contrast, posthypoxic reoxygenation results in massive mitochondrial superoxide formation, triggering subsequent formation of hydrogen peroxide (H₂O₂) and hydroxyl radical (OH•). Mycardial ischemia and reperfusion (I/R) injury has been shown to be attenuated by endogenous ROS scavenging systems, such as mitochondrial manganese superoxide dismutase, detoxifying O₂•⁻ and catalase or glutathione peroxidase, which inactivate H₂O₂.

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Less well studied is the thioredoxin / thioredoxin reductase system. Thioredoxins work through a -Cys-Gly-Pro-Cys-active site and supply electrons for the reduction of a wide variety of substrates, including peroxidases, which are critically in-
volved in ROS scavenging. Thioredoxin-1 (Txn1) and its corresponding reductase, thioredoxin reductase-1 (Txnrd1), are primarily localized in the cytosol, whereas thioredoxin-2 (Txn2) and its reductase, thioredoxin reductase-2 (Txnrd2), are both expressed in mitochondria. A third form, thioredoxin reductase-3, is mainly expressed in tests, and thus, has not been included in this study.

Txn1 has been demonstrated to participate in cardiac ROS scavenging and in limiting postischemic apoptosis and infarct size. By contrast, much less is known about the cardioprotective role of Txn2 and Txnrd2. The subcellular localization in mitochondria fueled speculation that Txn2 is central in the regulation of mitochondrial ROS in cardiomyocytes, given a solid 30% contribution of mitochondria to the cardiomyocyte cell volume. A potentially crucial role of Txn2 in modulating cardiac hypertrophy on stimulation by angiotensin 2 surfaces recently, consistent with the notion that the hypertrophy-driving O$_2$ and H$_2$O$_2$ force is controlled by Txn2.

Unfortunately, analysis of Txnrd1 and Txnrd2, which antagonize the myocardial ROS burst at the onset of postischemic reperfusion, was hampered by embryonic lethality of ubiquitous deletion of either reductase. To overcome this obstacle, we were able to generate mice with cardiomyocyte-specific Txnrd1 deletion, which were born at the normal Mendelian ratio and proved to be fully viable. In contrast, constitutive cardiomyocyte-restricted Txnrd2 deletion caused early postnatal death, prompting the generation of tamoxifen-inducible cardiac-specific Txnrd2-deficient mice. Taking advantage of cardiomyocyte-specific Txnrd1-/- and Txnrd2-deficient mice, we were able to define the role of mitochondrial versus cytosolic thioredoxin reductases in the scenario of I/R injury for the first time.

Materials and Methods

Heart-Specific Disruption of Txnrd1 and Txnrd2

All animal experiments were approved by the Bavarian Animal Care and Use Committee and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996. A proposed institution # 5637-001). Heart-specific inactivation of Txnrd1 was achieved by using the ML2a-Cre transgenic mouse line, as described. Tomato mice containing a fluorescence switch before (red, tomato) and after (green, eGFP) Cre activation (online-only Data Supplement Figure 1A) were kindly provided by Ralf Adams, Muenster, Germany. Mice overexpressing human thioredoxin-2 (Tg$^{H2}$) were created by Dr. Jiyoung Cai in collaboration with Dr. Dean P. Jones, Emory University, Atlanta, Georgia. Cardiac tissue-specific disruption of Txnrd2 was achieved by crossing Txnrd2$^{flox/flox}$ mice with a heart-specific tamoxifen-inducible Cre recombinase-expressing strain and feeding adult mice for 4 weeks with chow containing tamoxifen citrate ($\approx$ Txnrd2-Aic3, followed by 4 weeks of standard diet (Altromin, Lage, Germany) before I/R. For Txnrd1-/-ic and Txnrd2-/- mice, isolation of 4 weeks of tamoxifen citrate chow were followed by 2 weeks of standard diet.

Ischemia and Reperfusion

Ischemia of the heart was induced for 90 minutes by ligation of the left anterior descending coronary artery (LAD) as described. Reperfusion was then allowed for 24 hours or 14 days and followed by invasive monitoring of left ventricular function with a Millar tip catheter. The catheter was advanced through the left carotid artery and aorta into the left ventricle (LV) under continuous monitoring of the pressure curves until the diastolic pressure indicated localization in the LV. N-acetylcysteine (NAC, 15 mmoles/animal) was administered intraperitoneally 15 minutes before the onset of ischemia, and cyclosporin A (CsA, 10 mg/kg intravenously via tail vein) was administered at the onset of reperfusion.

Infarct Size, TUNEL Staining, Ultrastructural Analysis

Infarct size was determined with Evans blue exclusion under conditions of LAD reocclusion. A short axis matrix for resection, 3 short axis slices were incubated with triphenyl-tetrazolium-chloride (TTC) for 10 minutes. Microscopic pictures were taken and analyzed using planimetry (ImageJ). Nih, Bethesda, MD. TUNEL-positive cells were obtained from the ischemic region using Apoptag Kit (Millipore, Schwalbach, Germany) according to the manufacturer's guidelines. Nuclei were counterstained with DAPI (Vector Laboratories, Burlingame, CA).

For ultrastructural analysis, hearts were isolated and immediately fixed in 2.5% glutaraldehyde-2% paraformaldehyde (PFA). Samples were implanted in propylene and Polyembed 812 (Plano, Wetzlar, Germany). Ultrathin sections (60 nm) were stained with uranyl acetate and lead acetate and then examined on a Zeiss (Oberkochen, Germany) 902 electron microscope at 80 kV.

Embryonic Endothelial Progenitor Cells (eEPC) and Embryonic Stem (ES) Cell-Derived Cardiomyocytes

Vascular progenitor cells were derived from ubiquitous Txnrd2-/- embryonic (E7.75) according to a protocol established by Hatzopoulos et al. ES cell lines were established from E3.5 embryos from matings with Txnrd2$^{flox}$ and Txnrd2$^{flox/flox}$ mice. ES cell clones were stably transfected with the plasmid pCAG-35P-merCreMer by electroporation and selected by 1 µg/mL puromycin as described. Subsequently, MerCreMer-expressing ES cell clones were transfected with the plasmid pMHC-neo-PGK-Hyg (a kind gift from Loren Field, Indianapolis, IN). Cardiomyocyte selection was performed by hygromycin addition at d14 after leucokete inhibiting factor (LIF) withdrawal (initiating differentiation). Activation of Cre-recombinase and excision of the floxed Txnrd2 allele was performed by 4-hydroxytamoxifen addition for 72 hours (1 µmol/L). Hypoxia (<1% oxygen) was induced by superfusing cells with nitrogen for 18 hours before 4% reoxygenation in the case of cardiomyocytes, and for 6 hours before 2 hours’ reoxygenation with eEPCs. For fluorescence microscopy, DAPI (Vector Laboratories, Burlingame, CA) was used; for nuclear staining, troponin-1 and α-MHC antibodies (both Santa Cruz) were used.

Adult H.L.-1 Cell Knockdown of Thioredoxin Reductase-2

Small hairpin RNA against murine Txnrd2, we used 5’ATCGGAAGATCTCTAACTTTCGTCCTGACAGATTAAGGTGAGATTTCCTTCCCTTTTCTGCAGGGAGGATCCCTTTTTG-3’ (targeting 1187–1207bp in the Txnrd2-mRNA) and the scrambled sequence oligonucleotide 5’GA TCCGGGAATTGCTCTGAGCTTCTGCTGACAGATTAAGGTGAGATTTCCTTCCCTTTTCTGCAGGGAGGATCCCTTTTTG3’. Oligonucleotides were ligated into the pGreenPuro vector (System Biosciences). HL-1 cells were plated on fibronectin-coated plates and cultured with Claycomb media (Sigma) for transduction with lentivirus using a MOI of 1000/cell in the presence of 8 µg/mL Polybrein (Sigma). Puromycin (50 µg/mL) was added 48 hours after infection for another 48 hours. Hypoxia was induced for 12 hours (1% O$_2$) followed by reoxygenation (1 hour).

Protein Isoaltion, Protein Concentration Determination, and Immunoblotting

Protein isolation, protein concentration determination, and immunoblotting are described in detail in the online-only Data Supplement Methods.
Determination of Reduced Glutathione
Intracellular GSH levels were measured in Txnrd2-/- cells by high-performance liquid chromatography in normoxic cells and after hypoxia/reoxygenation, as described.30

Mitochondria: Isolation and Functional Analysis
Detailed methods are described in the online-only Data Supplement Methods. In brief, mitochondria were freshly isolated from Txnrd2-/-ic and control hearts by differential centrifugation according to standard protocols.31 Functional integrity of isolated mitochondria was routinely assessed with a Clark type oxygen electrode (Oxygraph™, Hansatech Instruments Ltd, UK). Permeability-transition–induced swelling of mitochondrial suspensions was measured by light scattering at 540 nm, as described recently.31 The generation of ROS was monitored by measuring the fluorescence of dichlorofluorescein with a SynergyHT2™ Multi-Mode Microplate Reader (Bio-Tek, Bad Friedrichshall, Germany) (Ex 485/20; Em 526/20).32 Superoxide production was measured enzymatically after conversion to H$_2$O$_2$ and expressed as H$_2$O$_2$ release from intact mitochondria as described.33 After I/R, aconitase activities (n=10 Txnrd2-/-, n=12 Txnrd2-/-ic, n=4 Txnrd1+/-, n=4 Txnrd1-/-ic, where n is the number of measurements; Cayman Chemicals, Ann Arbor, MI) were normalized to their normoxic counterparts (set to 100%), averaged, and subjected to statistical analyses.

Statistical Analysis
The results are given as mean±SEM. Statistical analysis of results among ≥2 experimental groups was performed with one-way analysis of variance (ANOVA). Whenever a significant effect was obtained with ANOVA, we performed multiple comparison tests among the groups using the Student Newman Kuel’s procedure. For comparing just two experimental groups, Student’s t test was applied. All procedures were performed with an SPSS statistical program (Version 19) after Shapiro Wilk and Levene testing for normality and equality of variances. Differences between groups were considered significant at P<0.05.

Role of Thioredoxin Reductase-1 Deficiency During Ischemia/Reperfusion
We first investigated the impact of Txnrd1-knockout on cardiac injury inflicted by ischemia (90 minutes) and reperfusion (24 hours). Immunoblotting of heart-specific Txnrd1 knockout mice (Txnrd1-/-) (Figure 1A) revealed a steep decrease of Txnrd1 in heart tissue (Figure 1B). Low Txnrd1 quantities were still detectable due to either mosaic expression of Cre in the cardiomyocytes or, most likely, the presence of noncardiomyocyte cells like vascular cells and fibroblasts, which also express Txnrd1. On I/R, Txnrd1-/- hearts differed from sham-operated experimental groups but did not notably differ from Txnrd1+/- hearts in their ability to maintain a residual LV function (LVDP after I/R: 82±4 mm Hg at 50 ng norepinephrine stimulation; vs 81±2 mm Hg at rest and 123±6 versus 114±4 mm Hg at 50 ng norepinephrine stimulation; no significant difference for each condition) (Figure 1C) (online-only Data Supplement Figure IB). Likewise, inducible Txnrd1-/- hearts, generated by cardiomyotropic rAAV2.9-Cre transduction of Txnrd1flox/flo mice (termed Txnrd1-/-icV) (online-only Data Supplement Figure IA) or wild-type C57BL/6 hearts did not alter postischemic hemodynamic function (online-only Data Supplement Figure IB).

Moreover, no statistically significant difference in the postischemic prevalence of TUNEL-positive cardiomyocytes was detectable between Txnrd1+/- and Txnrd1-/-icV mice (Figure 1D), although both levels significantly exceeded normoxic (sham) levels. Consistently, infarct size was not significantly different between Txnrd1+/- and Txnrd1-/-icV
hearts (online-only Data Supplement Figure IIA and IIB). These findings indicate that endogenous Txnrd1 does not fulfill an essential function in cardioprotection against oxidative stress as provided by postischemic reperfusion following 90 minutes’ LAD occlusion.

**Role of Thioredoxin-2 Overexpression During Ischemia/Reperfusion**

Next, we assessed postischemic cardioprotection in murine hearts overexpressing human Txn2 (TGhTxn2). Under basal conditions, no significant difference was obtained for LVDP in TGhTxn2 mice subjected to I/R compared with littermate controls, although under stimulation with 25 and 50 ng of norepinephrine (NE), cardiac performance increased in TGhTxn2 mice (LVDP 134 ± 6 versus 118 ± 4 mm Hg hearts at 50 ng of NE) (online-only Data Supplement Figure IIA). Similar results were obtained for contraction velocity dP/dt max (online-only Data Supplement Figure IIIB). These moderate effects may imply that, rather than the ROS scavenger Txn2, the corresponding reductase Txnrd2 might be of rate-limiting relevance during an I/R episode, prompting us to investigate cardiospecific Txnrd2-deficient hearts in this scenario.

**Role of Thioredoxin Reductase-2 Deficiency During Ischemia/Rerfusion**

We recently reported that ubiquitous inactivation and heart-specific disruption of Txnrd2 resulted in an embryonic and early postnatal lethal phenotype. In order to study I/R injury in mice lacking mitochondrial Txnrd2, we generated a mouse strain that lost Txnrd2-expression in cardiomyocytes on tamoxifen induction (Figure 2A and online-only Data Supplement Methods). An organ-specific decrease of Txnrd2 protein levels was evident in the heart, but not in the liver, using the C-terminal antibody 1C4 (TGhTxn2-Ic=inducible cardiac Txnrd2-Ic). Millar tip catheter analysis revealed a significant difference in left ventricular developed pressure (LVDP) at rest and with norepinephrine (NE) stimulation at 24 hours. Contraction velocity dP/dt max confirmed the systolic impairment (D), whereas the relaxation velocity dP/dt min was unchanged (E). F, For left ventricular end-diastolic pressure, no difference was found among experimental groups (P=0.14, 0.47, 0.42, and 0.27 at rest and with 10, 25, and 50 ng of NE). Txnrd2+/+ sham n=4, Txnrd2-Ic/+/+ sham n=5, Txnrd2+/+ I/R, and Txnrd2-Ic/+- I/R n=7. §P<0.05 versus Txnrd2+/+ I/R and Txnrd2-Ic/+- I/R. *P<0.05 versus all other groups.

**Figure 2.** Relevance of cardiospecific thioredoxin reductase-2 (Txnrd2) deletion for postischemic reperfusion injury. A, C57BL/6 mice were used, which carried a deleted and a floxed allele of Txnrd2 and a tamoxifen-inducible Cre under the control of the α-myosin heavy chain promoter. B, On tamoxifen induction, expression of Txnrd2 was largely reduced in the heart, but not in the liver, using the C-terminal antibody 1C4 (Txnrd2-Ic=inducible cardiac Txnrd2-Ic). C, Millar tip catheter analysis revealed a significant difference in left ventricular developed pressure (LVDP) at rest and with norepinephrine (NE) stimulation at 24 hours. Contraction velocity dP/dt max confirmed the systolic impairment (D), whereas the relaxation velocity dP/dt min was unchanged (E). F, For left ventricular end-diastolic pressure, no difference was found among experimental groups (P=0.14, 0.47, 0.42, and 0.27 at rest and with 10, 25, and 50 ng of NE). Txnrd2+/+ sham n=4, Txnrd2-Ic/+/+ sham n=5, Txnrd2+/+ I/R, and Txnrd2-Ic/+- I/R n=7. §P<0.05 versus Txnrd2+/+ I/R and Txnrd2-Ic/+- I/R. *P<0.05 versus all other groups.
Figure 3. *Txnrd2*−/−-*ic* aggravates cellular detriment of postischemic reperfusion injury. 

**A.** Electron beam microscopy examples of sham and ischemia/reperfusion (I/R) (60/30 minutes) treated hearts.

**B:** Quantification revealed that mitochondrial integrity (online-only Data...
124 ± 5 mm Hg), whereas the function at rest had recovered at this time point (online-only Data Supplement Figure IVB). The deterioration of systolic function in Txnrd2-/ic mice at 24 hours was mirrored in decay of contraction velocity dP/dt max (6424 ± 521 versus 8954 ± 365 mm Hg/s at rest, 13803 ± 1768 versus 18987 ± 1532 mm Hg/s with 50 ng of NE) (Figure 2D). Of note, diastolic function, assessed by relaxation velocity dp/dt min, was not significantly altered by cardiogenic loss of Txnrd2 (−6574 ± 457 versus −7519 ± 1645 mm Hg/s in controls, 12012 ± 1645 mm Hg/s in sham at rest) (Figure 2E). Moreover, no significant alteration in LVDP (Figure 2F) was obtained.

**Augmented Mitochondrial Impairment, Increased Cell Death, and Larger Infarct Size on Ischemia/Reperfusion in Thioredoxin Reductase-2 Deficient Hearts**

At a cellular level, transmission electron microscopy revealed losses of regularity of mitochondrial striation and of membrane integrity as early as 60 minutes after I/R in Txnrd2-/ic mice (Figure 3A), which exceeded the levels seen in Txnrd2+/+ hearts. A mitochondrial score consisting of swelling, loss of striation, and membrane disintegrity (online-only Data Supplement Data Figure V) confirmed this observation: although no significant difference in mitochondrial integrity was obtained in normoxic hearts, mitochondrial disintegration was obvious after I/R (Figure 3B). As assessed by the release of H2O2, heart mitochondria produced more ROS on addition of antimycin. Interestingly, comparably low amounts of H2O2 were observed for Txnrd2-/ic (−1768 versus 18897 ± 4 each) *P < 0.05 versus Txnrd2+/+. CsA, a well-documented mechanism of I/R injury is opening of mitochondrial mPTPs,35,36 which can be inhibited by the interaction of cyclosporin A (CsA) with the prolylserosine cyclophilin D located in the mitochondrial matrix. Notably, CsA application abolished the loss of systolic function after I/R in the Txnrd2-/ic but not in the Txnrd2+/+ hearts (Figure 4A and 4B). Moreover, CsA was also able to decrease the number of TUNEL-positive cells in Txnrd2-/ic hearts (Figure 4C) compared with healthy control mitochondria, a hallmark of mPTP-induced detriment, was also inhibited by CsA.

**Thioredoxin Reductase-2 Deficiency Sensitizes Toward Mitochondrial Pore Opening and Subsequent Cell Death**

A well-documented mechanism of I/R injury is opening of mitochondrial mPTPs,35,36 which can be inhibited by the interaction of cyclosporin A (CsA) with the prolylserosine cyclophilin D located in the mitochondrial matrix. Notably, CsA application abolished the loss of systolic function after I/R in the Txnrd2-/ic but not in the Txnrd2+/+ hearts (Figure 4A and 4B). Moreover, CsA was also able to decrease the number of TUNEL-positive cells in Txnrd2-/ic hearts (Figure 4C) compared with healthy control mitochondria, a hallmark of mPTP-induced detriment, was also inhibited by CsA.

**N-Acetylcysteine Rescues Postischemic Thioredoxin Reductase-2–Deficient Hearts**

In order to test the hypothesis that the antioxidant NAC might compensate for the impaired regeneration of oxidized mitochondrial thiols, we first employed cell culture assays. Because murine embryonic fibroblast cell cultures from Txnrd2-/ mice did not display a detectable difference in cell death on hypoxia and reoxygenation (data not shown), we developed 3 alternative cell culture models of Txnrd2 deficiency: (1) vascular progenitor cells derived from ubiquitous Txnrd2-/ / mice (eEPCs) (Figure 5A through 5C); (2) Txnrd2-deleted cardiomyocytes derived from Txnrd2flox/floxMerCreMer ES cells (Figure 5D through 5F); and (3) an adult murine cardio-
myocyte cell line (HL-1) in which Txnrd2 expression was experimentally decreased (Figure 5G and 5H).37

We analyzed the reduced glutathione pool after hypoxia/reoxygenation of eEPCs because glutathione (GSH) has been reported to be a valid surrogate parameter for thiol supply in various model organisms.38,39 As depicted in Figure 5B, NAC increased the GSH content in Txnrd2-/− eEPCs to levels found in untreated wild-type cells. Pretreatment with NAC caused a dramatic increase in cell survival of Txnrd2-deficient eEPCs after hypoxia and reoxygenation (Figure 5C) similar to pretreatment with the antioxidants GSH or α-tocopherol.

Analysis of ES cell-derived, contracting Txnrd2flx/foxMerCreMer cardiomyocytes (online-only Data Supplement Video I) was performed after 72 hours’ induction with tamoxifen when deletion of Txnrd2 expression was obtained (Figure 5D). Txnrd2-/−ES cell-derived cardiomyocytes staining positive for the cardiomyocyte-specific markers αMHC and actinin (Figure 5E) displayed an increased vulnerability toward hypoxia-reoxygenation stress, which was reversed to Txnrd2+/− levels by NAC application (Figure 5F, online-only Data Supplement Video II). A similar effect was obtained in HL-1 cardiomyocytes,37 displaying a reduced Txnrd2 expression after lentiviral transfection of Txnrd2-shRNA (Figure 5G). A gain, decreased Txnrd2 expression sensitized toward hypoxia-reoxygenation induced cell death unless NAC was supplied (Figure 5H).

To verify that NAC is capable of normalizing the phenotype of Txnrd2-/−ic in vivo, we applied NAC intraperitoneally 30 minutes before the onset of ischemia. In these animals, pretreatment with NAC improved LVDP and contraction velocity significantly 24 hours after ischemia. In fact, performance reached the functional levels of Txnrd2+/− mice treated with NAC (Figure 6A and 6B). NAC application even abolished the excess cell death (Figure 6C) and the mitochondrial detriment (Figure 6D and 6E) observed in postischemic Txnrd2-deficient hearts. Moreover, NAC was capable of reducing butyl-hydroperoxide-induced mitochondrial ROS burden to a large extent (Figure 6F).

**Discussion**

Recently, mutations in the mitochondrial Txnrd gene have been identified to cause dilated cardiomyopathy.40 In the present study, we provide evidence for an essential role of endogenous mitochondrial Txnrd2 in controlling I/R injury of the heart. To this end, we used mice in which exons 15–18 of the remaining Txnrd2 allele were flanked by Lox-P sites.23 On induction with tamoxifen, a cardiospecific Cre-recombinase translocated to nuclei and excised the remaining Txnrd2 allele. This strategy resulted in an approximately 70% loss of Txnrd2 expression in cardiac cells in vivo.

Such hearts subjected to LAD occlusion (90 minutes) and subsequent reperfusion displayed an early increase of mitochondrial swelling (Figure 3A and 3B), followed by enhanced cardiomyocyte cell death (Figure 3E and 3F) and an aggravated I/R-induced functional detriment (Figure 2C through 2F). An increased infarct size was found in Txnrd2-deficient hearts after 24 hours (Figure 3G and 3H) and up to 14 days of reperfusion (online-only Data Supplement Figure 4C). Moreover, the sensitivity of Txnrd2-deficient cells for hypoxia/reoxygenation-induced detriment was blunted by exog-
The enous application of the antioxidants glutathione, α-tocopherol, and NAC, the latter replenishing the reduced glutathione pool (Figure 5B and 5C). After peritoneal application, NAC was capable of abolishing not only the postischemic increase in the rate of myocardial cell death, but also the loss of cardiac function of Txnrd2-deficient mice (Figure 6A through 6D). Because the cysteine provider NAC is known to attenuate cellular vulnerability caused by a lack of thiol-based antioxidant systems, be it either glutathione or thioredoxin reductase-2, this NAC effect indicates that Txnrd2 deficiency significantly accentuates oxidative stress. Apparently, this phenomenon is not overcome by Txn2 upregulation in Txnrd2-deficient mice (Figure 2B), most likely due to the inability of mitochondria to regenerate oxidized Txn2 in the absence of Txnrd2.

Programmed cardiomyocyte death has evolved as a hallmark of cellular detriment during reperfusion, featuring direct caspase activation by cytochrome C released from...
Bax/Bak-permeabilized mitochondria. Moreover, postischemic reperfusion is sufficient to induce opening of the mitochondrial permeability transition pore (mPTP). ROS are known to contribute to the opening of the mPTP, triggering mitochondrial membrane potential (ΔΨm) breakdown, accumulation of fluids in the matrix, swelling and rupture of the mitochondrial outer membrane, and subsequent cell death. CsA, which inhibits cyclophilin D activity and subsequent mPTP opening, improved I/R injury in patients. In our models, CsA was able to reverse the increased sensitivity of Txnrd2-deficient hearts and cells toward mPTP opening to background levels (Figure 4A through 4D).

Because thiol supplementation via NAC or inhibition of mPTP opening by CsA prevents excessive I/R injury, it is reasonable to conclude that lack of Txnrd2 expression specifically impairs detoxification of ROS generated within mitochondria. In this context, it is noteworthy that cardiomyocyte-specific Txnrd1 deficiency did not affect the postischemic outcome (Figure 1). However, in a different model of ischemia (30 minutes), application of recombinant human Txn1 helped to reduce infarct size and apoptotic index. It appears likely that with increasing depth of I/R injury, mitochondrial Txnrd becomes more prominent as Txn regenerating enzyme. Supporting this notion, NAC as an exogenous thiol-providing ROS scavenger did not alter the functional state of postischemic Txnrd2+/+ control mice in our model. Consistently, in a recent clinical study, NAC (2 x 1200 mg/d) did not increase the myocardial salvage of patients with ST elevation myocardial infarction, bona fide Txnrd2 carriers. Only in the instance of Txnrd2 deficiency did thiol supplementation by NAC attenuate I/R injury, enabling a functional improvement to the level of Txnrd2+/+ control mice (Figure 6A and 6B).

In summary, we were able to demonstrate for the first time that cardiospecific deletion of Txnrd2 in the adult mouse heart induces an excessive vulnerability to I/R-induced injury. The underlying cellular mechanisms were increased mitochondrial ROS generation, impaired glutathione levels, increased sensitivity to mPTP opening, mitochondrial swelling, and, consequently, increased cell death and enhanced functional detriment. Rescue from Txnrd2 deficiency-induced cardiomyocyte injury, which was provided by N-acetylcysteine as well as CsA, points to the ability of Txnrd2 to block ROS-mediated mPTP opening in the advent of I and R of the heart. Taken together, our results highlight the role of the mitochondrial thioredoxin system in cardiomyocyte I/R injury.

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Disclosures

None.

References


The mitochondrial thioredoxin reductase gene (Txnrd2) in an adult mouse results in increased cardiomyocyte vulnerability during early postischemic reperfusion. In vivo, Txnrd2 deletion caused increased infarct size and cardiomyocyte apoptosis, whereas functional recovery of postischemic hearts was significantly impaired by the absence of Txnrd2. Of note, none of these hallmarks of reperfusion was obtained in mice lacking cytosolic thioredoxin reductase (Txnrd1). The observation that the mitochondrial permeability transition pore inhibitor cyclosporin A and the cysteine-rich antioxidant N-acetyl-cysteine were able to rescue the Txnrd2 phenotype indicates that indeed ROS scavenging and direct or indirect prevention of mitochondrial permeability transition pore opening are relevant mechanisms provided by endogenous mitochondrial thioredoxin reductase. Currently, it is unknown if functional impairment of Txnrd2, eg, by mutation, is clinically associated with increased myocardial ischemia-reperfusion injury, although a phenotype of dilated cardiomyopathy has been associated with mutations in Txnrd2.

**CLINICAL PERSPECTIVE**

Although clinical data on successful treatment of myocardial ischemia and reperfusion injury are scarce, the role of reactive oxygen species (ROS) caused by an imbalance of oxygen supply and proper mitochondrial utilization is well established. Cellular defense mechanisms against the reperfusion-induced wave of ROS, such as oxidation of reduced glutathione and thioredoxin, depend on regenerating enzymes in order to be available during the whole period of enhanced ROS formation.

In the current experimental study, we provide genetic evidence that deletion of mitochondrial thioredoxin reductase (Txnrd2) in an adult mouse results in increased cardiomyocyte vulnerability during early postischemic reperfusion. In vivo, Txnrd2 deletion caused increased infarct size and cardiomyocyte apoptosis, whereas functional recovery of postischemic hearts was significantly impaired by the absence of Txnrd2. Of note, none of these hallmarks of reperfusion was obtained in mice lacking cytosolic thioredoxin reductase (Txnrd1). The observation that the mitochondrial permeability transition pore inhibitor cyclosporin A and the cysteine-rich antioxidant N-acetyl-cysteine were able to rescue the Txnrd2 phenotype indicates that indeed ROS scavenging and direct or indirect prevention of mitochondrial permeability transition pore opening are relevant mechanisms provided by endogenous mitochondrial thioredoxin reductase. Currently, it is unknown if functional impairment of Txnrd2, eg, by mutation, is clinically associated with increased myocardial ischemia-reperfusion injury, although a phenotype of dilated cardiomyopathy has been associated with mutations in Txnrd2.