THIOREDOXIN REDUCTASE 2 IS ESSENTIAL FOR KEEPING LOW LEVELS OF H_2O_2 EMISSION FROM ISOLATED HEART MITOCHONDRIA

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Online Supplement

This section with supplementary material to the main text contains important information that complements, supports, and expands our study. We have divided this Supplement into two sections: Methods and Results. The first one comprises technical details that are important but not essential to the Methods section in the main text. The Results section is presented as figures with their captions that are sometimes extended to further explain the result observed.

Methods

Mitochondrial isolation from heart.

Mitochondria isolation and handling from guinea pig heart was performed as previously described (*1*) that when applied to mouse heart was slightly modified as follows. Proteinase (bacterial, type XXIV, Sigma) at 0.1mg/ml was added just before starting the homogenization procedure (no longer than 3 min for one or two mouse hearts). Three volumes of isolation solution (IS: 75 mM sucrose, 225 mM mannitol, and 1 mM EGTA at pH 7.4, containing 0.2% of fatty acid free BSA from Sigma) were quickly added after homogenization to block proteinase. After a first centrifugation (500 x *g* for 10 min) to discard unbroken tissue and debris, the supernatant was centrifuged at 10000 x *g* for 10 min to sediment the mitochondria, and washed twice thereafter by centrifugation at 7700 x *g* for 5 min each; the first one with IS in the presence of BSA, the second in its absence. In guinea pig and mouse hearts mitochondria Respiratory Control Ratios (RCR; state 3/state 4 respiration) of 10 or 5 respectively, were obtained using this method. Mitochondrial protein concentrations were determined using the bicinchronic acid method, BCA™ protein assay kit (Thermo Scientific, IL).

Results

Figure S1. Effect of AF on FET induced H_2O_2 emission in mitochondria from guinea pig.

Freshly isolated mitochondria (100-200μg mitochondrial protein) from guinea pig heart were pre-incubated in the absence or the presence of the indicated concentrations of Auranofin (AF) in the presence of the NAD^+-linked substrates glutamate/malate (G/M, 5mM each).

(A) Monitoring of mitochondrial H_2O_2 was performed with the Amplex Red (ARed) assay during state 4 and the transition from state 4 to state 3 of mitochondrial respiration (*1*). Shown are the normalized values of ARed fluorescence. Exogenous H_2O_2 (50 nM) was added for calibration purpose. Represented are the results of a typical experiment out of three independent ones.

(B) Kinetic characterization of AF-elicited increase in H_2O_2 emission from mitochondria. The inhibitory action of AF on mitochondrial TrxR2 was monitored from the increase in specific H_2O_2 emission from mitochondria under FET during state 4 (black squares) and state 3 (gray squares). The specific rates of ROS emission under states 4 or 3 were calculated from the slopes of H_2O_2 detected by the ARed assay (panel A), and using the internal calibration given by exogenous H_2O_2 in each assay. The $K_{0..5}$ was determined after non linear regression fitting of the experimental points with a hyperbolic, Michaelis-Menten type of equation.

Figure S2. Effect of AF on H_2O_2 emission from mitochondria under RET

Mitochondria (100-200μg mitochondrial protein) from GP heart were preincubated in the absence or the presence of the indicated concentrations of AF under 5 mM Succ. H_2O_2 was monitored with ARed (*2*); shown are the normalized values of ARed fluorescence. When indicated, 5 nM AF or 200 nM rotenone (Roten) were used. States 4 or 3 of mitochondrial respiration occur after addition of Succ or ADP (1 mM), respectively, which is indicated by arrows. Exogenous H_2O_2 (50nM) was added for calibration purpose. Represented are the results of a typical experiment out of three independent ones.

Previously, we demonstrated that H_2O_2 emission was 6-fold higher for Succ than for G/M during state 4 respiration (*1*). This was confirmed here by showing that AF further increased ROS emission in addition to RET alone. Interestingly, rotenone (200 nM) brought H_2O_2 levels to the same values observed under FET in the presence of 5 nM AF, suppressing both H_2O_2 emission by RET and the additional increase produced by AF. These results suggest that complex I in the respiratory chain is one of the main sources of ROS under RET, but not in FET because the activation in H_2O_2 emission due to AF is still present (see Fig. 2A, 2B in the main text). The observation that significant H_2O_2 emissions during state 3 following AF are still high is particularly striking. Previously, we demonstrated that ROS release in state 3 drastically

decreases both under FET (~2-fold) and RET (~12-fold) (*1*). Present data suggests that the TrxR2/Trx2 system is essential in order to keep low H_2O_2 emission from mitochondria, especially under state 3 respiration.

Figure S3. *In vitro* inhibition of Thioredoxin Reductase activity with Auranofin

Purified thioredoxin reductase from rat liver (Sigma, St. Louis, MO) was assayed at 25^{o}C in a mixture containing (in mM): 100 K_2HPO_4 buffer, pH 7.0, 10 EDTA, 5 DTNB, 0.24 NADPH, and 30nM TrxR (350mUnits; TrxR MW=58Kda). The enzyme activity was monitored with a spectrofluorometer (Photon Technology, Inc) λ_{exc} = 340 nm and λ_{em} = 450 nm under continuous stirring. (A) The reaction was triggered by NADPH addition to the assay mixture in the absence (Control) or the presence of the indicated concentrations of AF. (B) Extent of TrxR initial rate activity inhibition respect to the control in the absence of AF. The data points were fitted by non linear regression with a single exponential. IC_{50} is the amount of AF necessary to produce a 50% inhibition of TrxR activity. The arrow indicates the point at which AF and TrxR concentrations matched (30nM), which corresponded to 90% inhibition of the enzyme.

Figure S4. Confirmation of Prx3 dimer identity by DTT treatment and western blot

Mitochondrial suspensions were derivatized with AMS, precipitated with TCA, analyzed for Prx3 redox status by western blot as described under Experimental Procedures. Molecular weights (MW) were estimated using a 4-20% polyacrylamide gel and a logarithmic interpolation of standards using the Li-Cor Biosciences Odyssey software. In the absence of DTT, Prx3 resolved into 3 bands. The bottom band ran with an apparent MW of about 26 kDA, consistent with the monomeric form of Prx3 bearing an AMS moiety at each of the 3 cysteine residues. The top 2 bands appeared to be disulfide-linked dimeric forms of Prx3, probably with and without an AMS at the third cysteine. To confirm that the double band on top corresponded to Prx3 dimers, the samples were treated with 65mM DTT. The two top bands collapsed (~90%) into the monomeric form, but with an additional band with slightly lower MW (22kDa) than in the untreated sample. The lower MW band most likely reflects the fact that thiols formed upon reduction of the cross-linking disulfide would not be exposed to AMS under these conditions. Therefore, the DTT-sensitive top 2 bands represent disulfide-linked dimeric forms of Prx3.

Figure S5. Effect of catalase inhibition on H_2O_2 emission from mitochondria

Freshly isolated mitochondria from mouse heart (~100µg mitochondrial protein) were preincubated in the absence (control) or the presence of either 1mM or 2mM aminotriazole (AMT) alone or in the presence of 10nM AF under FET (see legend Fig. 1 and Methods). Monitoring of mitochondrial H_2O_2 emission was performed with the ARed assay; shown are the normalized values of ARed fluorescence. States 4 or 3 of mitochondrial respiration occur after addition of substrate (5mM G/M) or ADP (1mM), respectively, which is indicated by arrows. Exogenous H_2O_2 (50nM) was added for calibration purpose. Represented are the results of a typical experiment out of two independent ones.

Figure S1. Effect of AF on FET induced H_2O_2 emission in mitochondria from guinea pig

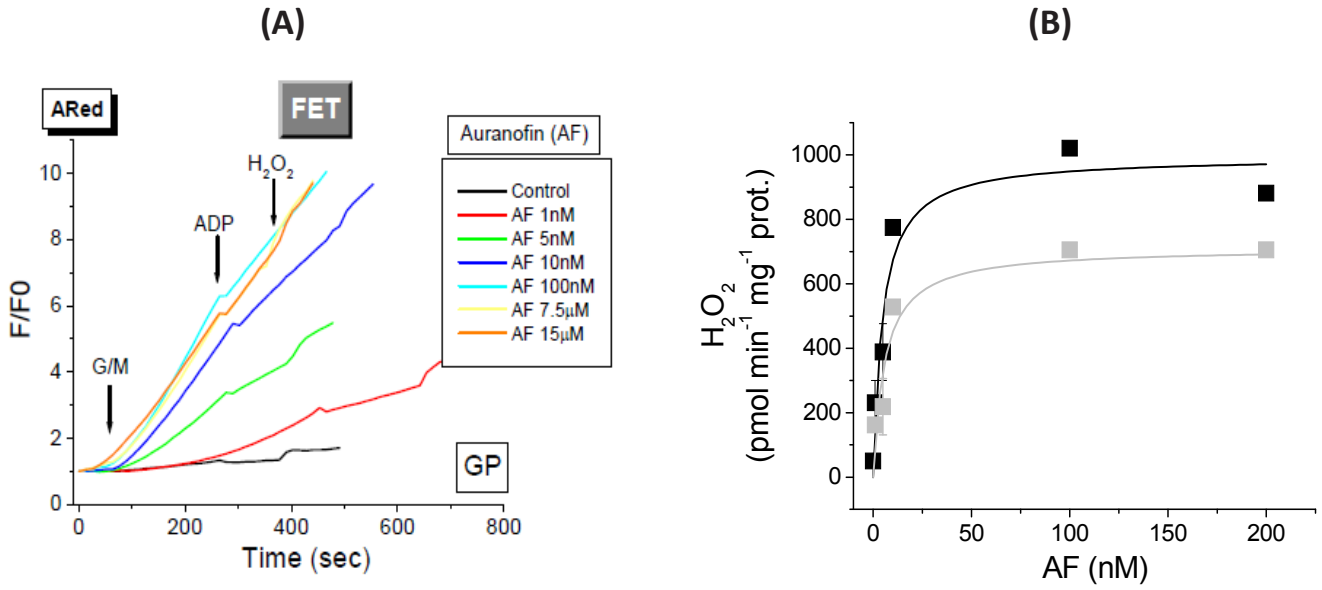


Figure S2. Effect of AF on H_2O_2 emission from mitochondria under RET

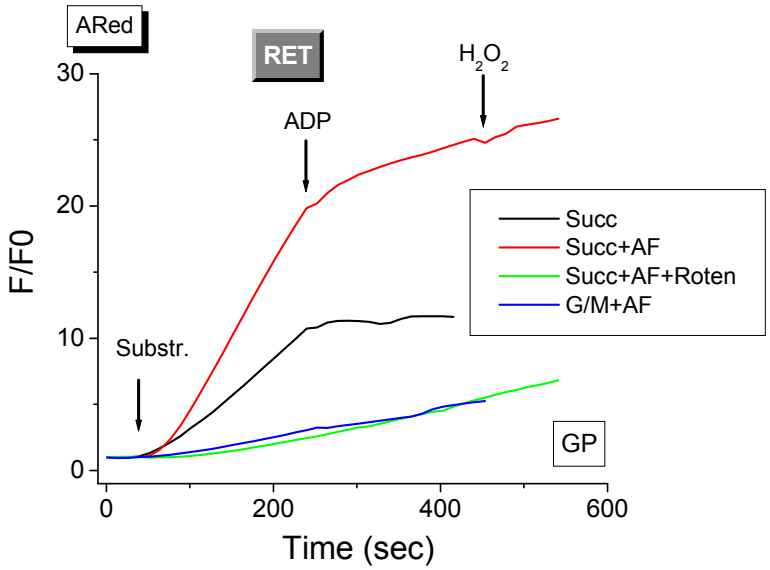


Figure S3. *In vitro* inhibition of Thioredoxin Reductase activity with Auranofin

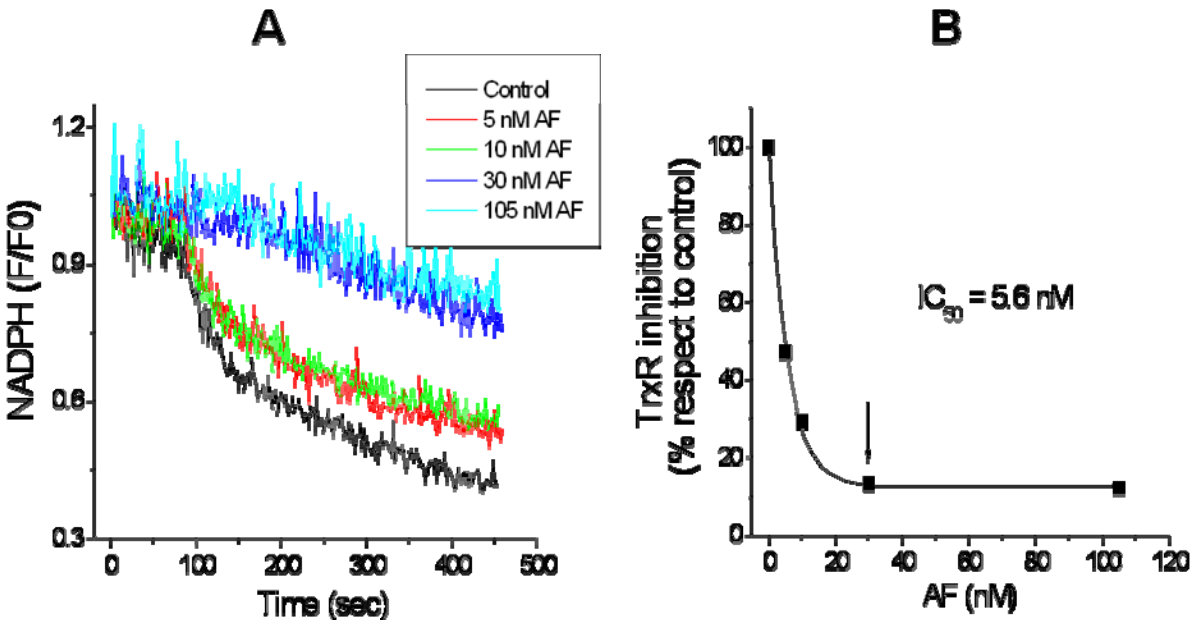


Figure S4. Confirmation of Prx3 dimer identity by DTT treatment and western blot

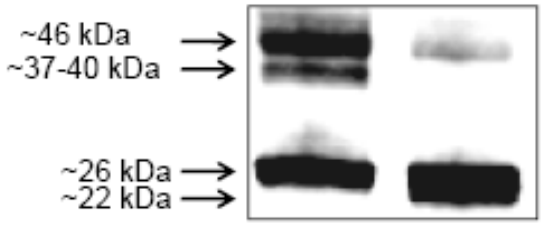
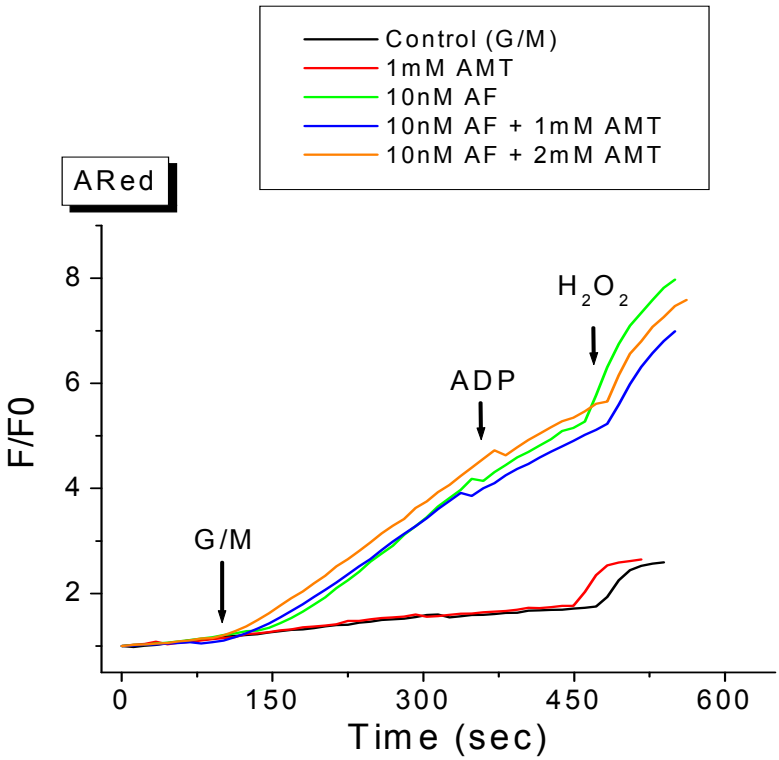


Figure S5. Effect of catalase inhibition on H_2O_2 emission from mitochondria



References

1. Aon, M. A., Cortassa, S., and O'Rourke, B. (2010) Redox-optimized ROS balance: A unifying hypothesis, *Biochimica et biophysica acta 1797*, 865-877.