Redox Imaging Using Cardiac Myocyte-Specific Transgenic Biosensor Mice


Rationale: Changes in redox potentials of cardiac myocytes are linked to several cardiovascular diseases. Redox alterations are currently mostly described qualitatively using chemical sensors, which however do not allow quantifying redox potentials, lack specificity, and the possibility to analyze subcellular domains. Recent advances to quantitatively describe defined redox changes include the application of genetically encoded redox biosensors.

Objective: Establishment of mouse models, which allow the quantification of the glutathione redox potential ($E_{\text{GSH}}$) in the cytoplasm and the mitochondrial matrix of isolated cardiac myocytes and in Langendorff-perfused hearts based on the use of the redox-sensitive green fluorescent protein 2, coupled to the glutaredoxin 1 (Grx1-roGFP2).

Methods and Results: We generated transgenic mice with cardiac myocyte–restricted expression of Grx1-roGFP2 targeted either to the mitochondrial matrix or to the cytoplasm. The response of the roGFP2 toward H$_2$O$_2$, diamide, and dithiothreitol was titrated and used to determine the $E_{\text{GSH}}$ in isolated cardiac myocytes and in Langendorff-perfused hearts. Distinct $E_{\text{GSH}}$ were observed in the cytoplasm and the mitochondrial matrix. Stimulation of the cardiac myocytes with isoprenaline, angiotensin II, or exposure to hypoxia/reoxygenation additionally underscored that these compartments responded independently. A compartment-specific response was also observed 3 to 14 days after myocardial infarction.

Conclusions: We introduce redox biosensor mice as a new tool, which allows quantification of defined alterations of $E_{\text{GSH}}$ in the cytoplasm and the mitochondrial matrix in cardiac myocytes and can be exploited to answer questions in basic and translational cardiovascular research. (Circ Res. 2016;119:1004-1016. DOI: 10.1161/CIRCRESAHA.116.309551.)

Key Words: angiotensin II ■ cytoplasm ■ diamide ■ ischemia ■ reactive oxygen species

The global redox status of a cell could be defined as the balance between oxidants and antioxidants. The concept of a global cell or tissue redox status, however, has been fine tuned in the past years at least, in part, through the application of biosensors, which allow dynamic, sensitive, and compartment- and redox-couple–specific analyses. The various cellular redox couples are maintained at nonequilibrium steady states that vary over time and from one cellular compartment to the next. Interestingly, they are somewhat independent of one another, although they all contribute to the overall balance of the redox state. The tripeptide glutathione (GSH) is a major cellular redox regulator. There is an increased recognition that changes in the cellular glutathione redox potential are important for cellular feedback and signaling pathways. Cardiac myocytes feature a defined organization of their cytoplasm containing the sarcomeric-contractile apparatus and their confined subcellular organelle compartments. The intracellular redox environment is assumed to differ between various organelles and the cytoplasm. Excitation contraction coupling is, thus, under tight redox control because it involves the interplay of different organelles with the cytoplasmic sarcoplasmic apparatus for calcium cycling and ATP supply. The possibility to quantify specific redox potentials in cardiac myocytes at these subcellular levels is challenging but could provide new insights into cardiac physiology. Up to now, it is still unclear (1) if subcellular compartments create redox microdomains in cardiac myocytes and (2) how they are spatially and temporally affected.
Two of the Grx1-roGFP1 cyto and Grx1-roGFP2 mito mouse lines were further characterized. We used adult male and female animals (at the age of 8–14 weeks) for our experiments. All animal work conformed to institutional guidelines and was approved by the Niedersächsische Landesamt für Verbraucherschutz und Lebensmittelsicherheit (approval number 3392–42502–04–13/1208). Founder mice and their resulting heterozygous offspring were genotyped by a standard polymerase chain reaction using the primers: 5'-CCCTCCTTTCCTCTGCACAG-3' and 5'-ATAGACTGAGGACCCGT-3', resulting in a 500-bp fragment for Grx1-roGFP2 cyto and 710-bp for Grx1-roGFP2 mito mouse lines on a gel. Positive founder mice were mated with wild-type (WT) animals to produce heterozygous offspring and WT littermate controls. The oldest animals, which were analyzed so far, had an age of 60 weeks without any signs of premature death, impaired heart function, or loss of Grx1-roGFP2 expression.

**Myocardial Infarction**

Myocardial infarction (MI) was achieved by permanent ligation of the left anterior descending artery as described previously.6

**Echocardiography**

Echocardiography and measurement of left ventricular dimension in systole, left ventricular dimension in diastole, fractional area shortening, anterior and posterior wall thickness, and ejection fraction were performed as described.15 In brief, mice were anesthetized using 1% isoflurane (Forene, Abbott) and 2-dimensional images, and M-mode tracings were recorded from the parasternal long- and short-axis view at midpapillary level (Vevo 2100 system, VisualSonics Inc, Toronto, Canada). Fractional area shortening and ejection fraction were used as marker for cardiac contractile function.

**Isolation of Cardiac Myocytes**

Adult ventricular cardiac myocytes were isolated via the Langendorff perfusion. Mice were euthanized, and the hearts were removed and quickly transferred into a chamber filled with ice-cold PBS, where the aorta was tied to a 21G cannula. The heart was then perfused at 37°C with Ca2+-free perfusion buffer (in mmol/L: NaCl 113, KCl 4.7, KH2PO4 0.6, NaHPO4×2H2O 0.6, MgSO4×7H2O 1.2, NaHCO3 12, KHCO3 10, HEPES 10, Tauroine 30, 2,3-butanedione-monoxime 10, glucose 5.5, and pH 7.4) for 3 minutes. To digest the heart, it was then perfused with 30 mL digestion buffer containing liberase dispase high concentration (0.04 mg mL−1, Roche), trypsin (0.025%, Gibco), and CaCl2, 12.5 μmol/L. Afterward, the atria were carefully excised and discarded; the digest ventricles were dissected for 30 s in 2.5 mL digestion buffer. To stop the digestion, 2.5 mL stop buffer I (perfusion buffer containing 1% BSA [Sigma] and 50 μmol/L CaCl2) were added to the cell suspension, which was then homogenized for 3 minutes using a 1 mL syringe without a needle. Ten minutes after sedimentation, the cardiac myocyte pellet was transferred into stop buffer II (perfusion buffer containing 0.5% BSA and 37.5 μmol/L CaCl2) for gradual recalcification ≤1 mmol/L of calcium. The cardiac myocytes were plated onto round laminin (Sigma)-coated coverslides (24 mm; Thermo Scientific) and incubated at 37°C and 5% CO2 until use.

**Mitochondrial Fluorescence Staining of Cardiac Myocytes**

Mito-tracker (Life Technologies) was diluted 1:10:000 in the perfusion buffer containing 1 mmol/L of Ca2+; 500 μL of the mito-tracker solution was added to each cardiac myocyte containing glass coverslip and was incubated for 20 minutes at 37°C. The cardiac myocytes were then washed 3× with PBS followed by a fixation with 4% PFA for 15 minutes at room temperature. After washing the cells 3× with PBS, the cells were then mounted onto the glass slides using Fluormount mounting medium (Sigma). The cells were then imaged using the LSM 510 Meta confocal laser scanning microscope (Carl Zeiss, Jena, Germany) equipped with a 63× Plan-Neofluar 1.3NA water-corrected objective. For close-ups, 6 z-series optical sections were collected with a step-size of 0.05 μm. Z-series are displayed as maximum z-projections. Gamma, brightness, and contrast were adjusted (identically for compared image sets) using Adobe Photoshop CS2.
Redox Measurements of Isolated Cardiac Myocytes

Isolated cardiac myocytes plated onto laminin-coated glass coverslips were incubated for at least 45 minutes before imaging. Then, the coverslips were mounted in the imaging chamber and washed once with 400 µL of imaging buffer (in mmol/L: NaCl 144, KCl 5.4, MgCl₂ 1, CaCl₂ 1, HEPES 10, and pH 7.3) at room temperature. The redox measurements were performed using the inverted fluorescence microscope IX83 (Olympus) and Visview software. The roGFP2 sensor was excited at 488 and 405 nm using a Polychrome V light source (Till Photonics). The emitted light from the sample was detected via a CCD camera (emission filter 510±15 nm). An exposure time of 10 ms usually led to a good signal:noise ratio, and images were acquired in GFP emission channels every 5 s; 400 µL of the desired compound solution including H₂O₂, dithiothreitol, isoprenaline, or angiotensin II were added into the chamber as soon as the 405/488 nm ratio reached a stable baseline.

Hypoxia

For hypoxia experiments, the inverted IX83 fluorescence microscope (Olympus) was equipped with the cellVivo incubation setup (Pecon) allowing to control temperature (37°C) and gas mixture (0.1%–20% O₂ and 5% CO₂).

Calculation of the E°roGFP redox Potentials

Oxidation difference (OxD) and E°roGFP calculations were performed as described in the studies by Meyer and Dick and Morgan et al. Determining the E°roGFP values in basal conditions requires the analyses of the fluorescence intensities at 405 and 488 nm after stimulation with H₂O₂ or diamide (maximum oxidation response) and dithiothreitol (maximum reduction response). On the basis of these values, the OxD of the probe was calculated. The OxD is the ratio of the number of oxidized molecules to the total number of molecules (OxD roGFP2=[roGFP2ox]/[roGFP2red+roGFP2ox]). In short, the emission intensities (I) obtained from the measurements at 405 and 488 nm were used to calculate the OxD. The OxD then was applied to calculate the probe redox potential, where E° roGFP2 is −280 mV. Assuming that the probe and the glutathione redox couple are in equilibrium, the E°roGFP2 values were measured with standardized microcopy settings, including laser intensities and exposure times. Calculation intensities (I) obtained from the measurements at 405 and 488 nm were used to calculate the OxD. The OxD then was applied to calculate the probe redox potential, where E°roGFP2 is −280 mV. Assuming that the probe and the glutathione redox couple are in equilibrium, the E°roGFP2 values were measured with standardized microcopy settings, including laser intensities and exposure times.

\[
\text{OxD}_{\text{roGFP}} = \frac{405 \times 1488 \text{red} - 405 \times 1488 \text{ox}}{1405 \times 1488 \text{red} - 1045 \times 1440 \text{ox} + 1405 \times 1488 - 1405 \times 1488}
\]

\[
E_{\text{roGFP}} = E_{\text{roGFP}^2} + \frac{RT}{2F} \ln \left( \frac{1 - \text{OxD}_{\text{roGFP}}}{\text{OxD}_{\text{roGFP}}} \right)
\]

The pH-corrected E°roGFP was calculated according to the following equation as described.\(^{17}\)

\[
E_{\text{roGFP}^2} = E_{\text{roGFP}} - 60.1 \text{mV} \times (pH - 7)
\]

Dividing the highest ratio of the H₂O₂ response by the lowest ratio of the dithiothreitol response was used to determine the dynamic range of the biosensor in the settings applied.

Whole-Heart Imaging

Mice were euthanized and a thoracotomy was performed. The heart was rapidly excised and immersed in a cold bath of Tyrode solution (in mmol/L: NaCl 128.3, KCl 4.7, CaCl₂ 1.36, MgCl₂ 1.05, NaHCO₃ 20.2, NaH₂PO₄ 0.42, and glucose 10). A 2.1G cannula was inserted into the aorta and knotted with silk suture allowing retrograde perfusion in the Langendorff mode. To this end, the cannula was connected to a constant-pressure perfusion system with Tyrode solution warmed to 37°C and equilibrated with 95% O₂ and 5% CO₂. The Langendorff-perfused whole-heart imaging setup was established using a stereo microscope, 1.5x zoom (SMZ1500, Nikon). The redox measurements were performed using a polychrome light source (Till Photonics) controlled by the Visiview software. The roGFP2 sensor was excited at 488 and 405 nm, and the emitted light from the sample was detected via a CCD camera at 510 nm. An exposure time of 30 ms usually led to a good signal:noise ratio and images were acquired every 3 s. The Tyrode solution containing the desired compound solution including H₂O₂ and dithiothreitol was perfused through the heart as soon as the 405/488 nm ratio reached a stable baseline. Mean intensities at 405/488 nm were used to calculate the OxD as described above.

Mitochondrial Isolation

Heart tissue was sliced into small pieces, minced in 20 mmol/L HEPES pH 7.6, 220 mmol/L mannitol, 70 mmol/L sucrose, 1 mmol/L EDTA, and 0.5 mmol/L phenylmethylsulfonyl fluoride and homogenized in a motor-driven potter at 500 rpm for 25x. Debris were spun down at 800g for 15 minutes at 4°C. The supernatant was stored (homogenate 1) and the process was repeated with the pellet (homogenate 2). Homogenates 1 and 2 were pooled and centrifuged at 800g for 30 minutes at 4°C to sediment residual debris. The supernatant was transferred into fresh tubes, and the mitochondria were sedimented at 1000g for 10 minutes at 4°C. After an additional washing step, the mitochondrial pellet was resuspended and mitochondrial protein concentration was determined using the Bradford assay.

Blue Native PAGE Analysis

For blue native PAGE analyses, mitochondria were solubilized in digitonin-containing buffer as described previously to a final concentration of 1 µg/mL and incubated on ice for 20 minutes. Debris were removed by centrifugation (20000g, 15 minutes, 4°C). The supernatant was mixed with 10x blue native-loading dye, incubated on ice for 5 minutes and complexes separated on 2.5% to 10% (complex I and complex IV) or 4% to 13% (complex II, complex V, and Complexes I and IV) polyacrylamide gradient gels as described. In gel activity, staining was performed as described previously.

Oxygen Consumption Rate

Oxygen consumption rate of isolated mitochondria was analyzed in the Seahorse XF96 extracellular flux analyzer (Seahorse Bioscience, Billerica, MA). Mitochondria (500 ng) were given into a chilled XF cell culture plate. The plate was centrifuged at 2000g for 20 minutes at 4°C to sediment the mitochondria. The volume of each well was filled up to a final volume of 180 µL per well with mitochondrial assay solution buffer (70 mmol/L sucrose, 220 mmol/L mannitol, 2 mmol/L HEPES, 10 mmol/L KH₂PO₄, 5 mmol/L MgCl₂, 1 mmol/L EGTA, and 0.2% BSA). The plate was incubated for 30 minutes at 37°C without CO₂. Oxygen consumption was then analyzed after sequential addition of 10 mmol/L succinate, 4 mmol/L ADP, 2 µmol/L rotenone, and 2 µmol/L antimycin A.

Statistics

For the roGFP2 measurements, the baseline intensities obtained for each excitation wavelength was normalized to 1. Then, the ratio was calculated for the normalized intensities. Data are presented as mean±SEM. Statistical analyses were performed using Student 2-tailed t test. One-way ANOVA analysis (Bonferroni post hoc test) was performed in cases of comparisons with more than 2 groups. Values of P<0.05 were considered statistically significant. Every redox potential calculation is based on n-number of independent isolations/mice as indicated in the figure legends.

Results

Transgenic Expression of Grx1-roGFP2 in Cardiac Myocytes Does Not Affect Heart Function

We developed cardiac myocyte–specific Grx1-roGFP2 transgenic mouse models, which enable to determine the E°roGFP2 in the cytosol or mitochondrial matrix in isolated cardiac myocytes and the heart. The Grx1-roGFP2 is restricted to cardiac myocytes in these mouse lines because of the α-myosin heavy chain promoter-driven expression of the biosensor (Figure 1A). Mitochondrial matrix localization of the sensor in cardiac myocytes was enabled by the use of a signal.
Figure 1. Transgenic mice with cardiac expression of the reduction–oxidation-sensitive green fluorescent protein (roGFP)2 localized either to the mitochondria or to the cytosol. A, Schematic drawing of the plasmid DNAs used for the generation of the Grx1-roGFP2 mice. Targeting to the mitochondrial matrix was achieved by the signal sequence from Neurospora crassa ATP synthase protein 9 (atp9). B, Overlay of bright-field and fluorescence images of cardiac myocytes isolated from cyto1, cyto2, mito1, mito2, and wild-type (WT) mice. C, Quantification of GFP-positive cardiac myocytes from the indicated mouse lines. For each mouse line, values of 3 mice were included; >50 cardiac myocytes per mouse were quantified, mean±SEM. D, Confocal microscopy in isolated cardiac myocytes derived from the indicated mouse lines after staining with red mito-tracker. Echocardiography of 4 to 7 mice per genotype and respective WT mice was performed at the age of 8 wk. E, Fractional area shortening (FAS), (F) ejection fraction (EF), (G) posterior wall thickness (PWTh), and (H) anterior wall thickness (AWTh), mean±SEM. I, Isolated mitochondria from the indicated mouse lines were analyzed for mitochondrial complex I, II, IV, and V activity by blue native PAGE analysis. αMHC indicates α-myosin heavy chain; and Grx1, glutaredoxin 1.
sequence from Neurospora crassa ATP synthase protein 9. 7 founder lines for the Grx1-roGFP2 cyto mice and 4 founder lines for the Grx1-roGFP2 mito mice positive for expression of the fluorescent sensor proteins were selected. We performed in-depth analysis of 2 independent founder lines, in which the Grx1-roGFP2 biosensor is localized in the cytosol (cyto1 and cyto2), and of 2 founder lines, in which the sensor is targeted to the mitochondrial matrix (mito1 and mito2). All had an unequivocal expression of roGFP2 in cardiac myocytes (Figure 1B and 1C). Cardiac myocyte–specific expression was verified in cryosections (Online Figure 1A), the lack of fluorescence in isolated cardiac fibroblasts (Online Figure 1B), and via immunohistochemistry stainings using anti-GFP antibodies (Online Figure 1A). Hearts of the transgenic mice did not show any obvious differences to their WT littermates when analyzed by Trichrome staining (Online Figure 1B through 1D). The roGFP2 localized to the mitochondria in the cardiac myocytes isolated from the Grx1-roGFP2 mito mice as determined by staining with mito-tracker and confocal microscopy, whereas no colocalization was observed in the Grx1-roGFP2 cyto cardiac myocytes (Figure 1D; Online Figure 1A). Mitochondrial localization was additionally verified by fluorescence intensity measurements in isolated cardiac myocytes and mitochondria (Online Figure 1IA and 1IC). Cardiac myocytes from all transgenic mouse lines had significantly higher fluorescence signals at 405 and 488 nm compared with WT cardiac myocytes. In isolated mitochondria, however, significant fluorescence intensity was detected in the samples of the Grx1-roGFP2 mito mice only. Fractional area shortening, ejection fraction, and anterior and posterior wall thickness were determined by echocardiography in the transgenic mouse lines and their respective WT littermates and excluded cardiotoxicity (Figure 1E through 1H; Online Figure 1ID). We additionally excluded mitochondrial damage by the transgenic expression of the biosensor by analyzing mitochondrial respiratory complex I, II, IV, and V activities (Figure 1f; Online Figure 1VA) and oxygen consumption rate of isolated mitochondria (Online Figure 1VB).

**Dynamic Grx1-roGFP2 Response to H₂O₂ and Dithiothreitol in Transgenic Cardiac Myocytes**

roGFPs allow real-time visualization of the response toward an oxidative or reductive stimulus based on the conformational shift on reduction or oxidation (Figure 2A). Disulfide formation between the cysteine residues on H₂O₂ treatment promotes protonation of the chromophore and increases the excitation spectrum peak near 400 nm at the expense of the peak near 490 nm, whereas the opposite response can be observed on response to a reduction stimulus, such as dithiothreitol (Figure 2B through 2E; Online Movies 1 and II). We determined the ratios of fluorescence excitation at 405 and 488 nm in isolated cardiac myocytes, which accordingly indicate the extent of oxidation or reduction while canceling out the amount of indicator and the absolute optical sensitivity based on the ratiometric nature of the measurements. Isolated cardiac myocytes from cyto1, cyto2, mito1, and mito2 mice were treated with a single bolus of 100 μmol/L H₂O₂, 1 mmol/L dithiothreitol. After application of dithiothreitol, we observed a significant decrease in the 405/488 nm ratio (Figure 2F), whereas addition of H₂O₃ resulted in an increase demonstrating that the basal Eₘ₉₈ in the cytosol and the mitochondrial matrix lies within the effective range of the biosensor. The changes occurred rapidly to both stimuli in the cyto and mito cardiac myocytes and reached a stable plateau within <100 to 200 s, which indicates that the biosensor in the transgenic animals indeed dynamically reports real-time oxidation or reduction responses. To demonstrate that the sensor also responds similar to a subsequent oxidation and reduction stimulus, we treated cyto1 and mito1 cardiac myocytes with a single bolus of 100 μmol/L H₂O₂ followed by a washing step and a subsequent treatment with 1 mmol/L dithiothreitol (Online Figure 5). In line with the treatment with either H₂O₂ or dithiothreitol alone, the subsequent stimulation with H₂O₂ and dithiothreitol was responded with a significant increase followed by a significant decrease of the 405/488 nm ratio, respectively. The extent of the response to both stimuli was comparable to the single treatments. As we observed a higher rate of cell death after the subsequent stimulation with H₂O₂ and dithiothreitol compared with almost no cell death after single bolus treatment, we chose the single bolus stimulations in the following experiments.

**Redox Compartmentalization in the Mitochondria and the Cytosol**

Genetically encoded redox biosensors permit titration and the quantification of redox potentials of a defined redox pair. We titrated the dose–response of the Grx1-roGFP2 biosensor in cardiac myocytes isolated from the cyto1 and mito1 mice by exogenous application of 1 to 500 μmol/L H₂O₂ (Figure 3A) and 0.02 to 3 mmol/L dithiothreitol (Figure 3B). H₂O₂ affects the GSH:GSSG ratio in a cell and, thus, via the Grx1 indirectly the roGFP2. To gain insight, if the applied H₂O₂ concentrations were sufficient to achieve the maximum oxidation of the roGFP2, isolated cardiac myocytes were also treated with 1 to 500 μmol/L diamide (Figure 3C). Diamide is a sulfhydryl reagent that oxidizes the sulfhydryl groups of the roGFP2 to the disulfide form directly and, thus, is independent from the endogenous glutathione pool. The maximum responses toward H₂O₂ versus diamide were almost the same indicating that the applied H₂O₂ concentration was indeed sufficient to reach maximum oxidation. Starting from 100 μmol/L H₂O₂, 100 μmol/L diamide, and 2 mmol/L dithiothreitol, the oxidation and reduction responses reached their maxima in the cyto1 and the mito1 cardiac myocytes. The extent of the maximum responses to these H₂O₂, diamide, and dithiothreitol concentrations, however, differed significantly in the cytosol versus the mitochondrial matrix, indicating distinct basal Eₘ₉₈ in both compartments. Whereas the cytosol responded with higher deviations after addition of dithiothreitol compared with the mitochondrial matrix, the opposite was observed after addition of H₂O₂ and diamide. The same effect in the response after treatment with H₂O₂ or dithiothreitol was observed in the 2 other independent founder lines, that is, cyto2 and mito2 (Online Figure VI).

To gain insight into the sensitivity of the sensor, we determined the dynamic range, which reached values of 4.8, 4.3, 5.1, and 4.2 for the Grx1-roGFP2 cyto1, Grx1-roGFP2 cyto2, Grx1-roGFP2 mito1, and Grx1-roGFP2 mito2 cardiac
myocytes, respectively. These values are in accord with values described for roGFP2 in other settings and allow to detect even small changes in the redox potential.

We next calculated the $E_{\text{GSH}}$ in the cytosol versus mitochondrial matrix based on the $OxD_{\text{roGFP2}}$ of the maximum response to $H_2O_2$ versus dithiothreitol or diamide versus dithiothreitol (Table). We observed a stable $E_{\text{GSH}}$ of cardiac myocytes in the cytosol and the mitochondria with a significantly more oxidized environment in the cytosol. The intracellular pH differs in various subcellular compartments. In the roGFP2 molecule, the barrel structure is fully intact and, thus, effectively shielded from the environment. This rationalizes why the fluorescence ratio of roGFP is not significantly affected by pH changes within the physiological range.

Figure 2. Response of isolated Grx1-roGFP2 cardiac myocytes to $H_2O_2$ and dithiothreitol (DTT). A, The reduction–oxidation-sensitive green fluorescent protein (roGFP)2 contains 11 β-strands arranged to a barrel structure. Engineering of 2 surface-exposed cysteines (Cys204 and Cys147) on the 2 adjacent β-strands 7 and 10 in position allowing reversible disulfide formation on oxidation exploits a structure-dependent shift in the protonation status of the chromophore for ratiometric measurements. Fluorescence excitations of isolated cardiac myocytes derived from a mito1 mouse at 405 and 488 nm after a single bolus of 1 mmol/L DTT (B and C) or 100 µmol/L $H_2O_2$ (D and E). F, Ratio of the fluorescence excitations at 405/488 nm of cardiac myocytes isolated from the indicated mouse lines after a single bolus treatment with 1 mmol/L DTT or 100 µmol/L $H_2O_2$. Grx1 indicates glutaredoxin 1.
in contrast, for example, to the cp yellow fluorescent protein (YFP)-derived probe HyPer. The pH still has to be considered when determining the $E_{\text{GSH}}$. To this end, we calculated the $E_{\text{GSH}}$ adjusted to the estimated compartment pH assuming a pH of 7.4 for the cytoplasm and 7.91 for the mitochondrial matrix. The pH-corrected values demonstrated comparable to the noncorrected values, a more reduced environment in the mitochondrial matrix versus the cytoplasm.

$E_{\text{GSH}}$ calculations were repeated with cardiac myocytes incubated in calcium-free buffer or addition of 10 mmol/L glucose. Omitting calcium or adding glucose did not affect the $E_{\text{GSH}}$. Because all measurements were performed with freshly isolated cardiac myocytes, we additionally tested the $E_{\text{GSH}}$ in cardiac myocytes, which were cultured overnight after isolation. The $E_{\text{GSH}}$ of the overnight cultured cardiac myocytes were not significantly different compared with the freshly isolated cells.

**Imaging in Langendorff-Perfused Hearts**

To gain insight into the $E_{\text{GSH}}$ in cardiac myocytes within the tissue context, we established ratiometric measurements of whole hearts using a stereo microscope and polychrome light source in combination with a CCD camera (Figure 4A). The heart was retrograde perfused in the

![Figure 3. Grx1-roGFP2 responses to H$_2$O$_2$, dithiothreitol (DTT), and diamide in the cytosol and mitochondrial matrix compartment.](http://circres.ahajournals.org/) Cardiac myocytes isolated from cyto1 and mito1 mice were treated with 1 to 500 mmol/L H$_2$O$_2$ (A), 0.02 to 3 mmol/L DTT (B), or 1 to 500 mmol/L diamide (C). Ratios of the fluorescence excitations at 405/488 nm were recorded. Measurements of cardiac myocytes from 15 (cyto1, H$_2$O$_2$), 17 (cyto1, DTT), 11 (cyto1, diamide), 18 (mito1, H$_2$O$_2$), 15 (mito1, DTT), and 13 (mito1, diamide) mice were included, mean±SEM. *P<0.05 (compared with nontreated control), 1-way ANOVA analysis, Bonferroni post hoc test). Grx1 indicates glutaredoxin 1; and roGFP, reduction-oxidation-sensitive green fluorescent protein.

**Table. $E_{\text{GSH}}$ of Isolated Cardiac Myocytes Derived From the Indicated Mouse Lines**

<table>
<thead>
<tr>
<th>Redox Potential</th>
<th>Cyto1</th>
<th>Cyto2</th>
<th>Mito1</th>
<th>Mito2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diamide–DTT</td>
<td>$-257.2\pm0.7$ mV (102)</td>
<td>$-255.8\pm0.6$ mV (154)</td>
<td>$-278.9\pm0.4$ mV (108)</td>
<td>$-277.1\pm0.4$ mV (97)</td>
</tr>
<tr>
<td>H$_2$O$_2$–DTT</td>
<td>$-254.8\pm0.8$ mV (112)</td>
<td>$-256.3\pm0.7$ mV (154)</td>
<td>$-278.9\pm0.4$ mV (104)</td>
<td>$-275.4\pm0.4$ mV (98)</td>
</tr>
<tr>
<td>Diamide–DTT pH corrected</td>
<td>$-279.3$ mV (pH 7.4)</td>
<td>$-280.8$ mV (pH 7.4)</td>
<td>$-334.7$ mV (pH 7.91)</td>
<td>$-331.3$ mV (pH 7.91)</td>
</tr>
<tr>
<td>H$_2$O$_2$–DTT pH corrected</td>
<td>$-281.8$ mV (pH 7.4)</td>
<td>$-280.4$ mV (pH 7.4)</td>
<td>$-334.4$ mV (pH 7.91)</td>
<td>$-333.0$ mV (pH 7.91)</td>
</tr>
<tr>
<td>Calcium-free buffer</td>
<td>$-254.8\pm1$ mV (42)</td>
<td>$-255.5\pm0.8$ mV (33)</td>
<td>$-274.2\pm0.8$ mV (30)</td>
<td>$-274.8\pm0.7$ mV (28)</td>
</tr>
<tr>
<td>+10 mmol/L glucose</td>
<td>$-258.5\pm0.8$ mV (27)</td>
<td>$-255.5\pm0.9$ mV (25)</td>
<td>$-273.1\pm1$ mV (18)</td>
<td>$-276.9\pm0.9$ mV (35)</td>
</tr>
<tr>
<td>Overnight culture</td>
<td>$-255.8\pm2$ mV (16)</td>
<td>$-255.4\pm2$ mV (27)</td>
<td>$-277.4\pm1$ mV (28)</td>
<td>$-271.1\pm0.6$ mV (47)</td>
</tr>
</tbody>
</table>

Numbers in the parentheses indicate the number of cardiac myocytes analyzed, mean±SEM. DTT indicates dithiothreitol.

*P<0.05 (compared with the respective condition analyzed in the mito1 and mito2 cardiomyocytes), 1-way ANOVA (Bonferroni post hoc test).
Langendorff mode. H$_2$O$_2$ or dithiothreitol were subsequently added to the Tyrode solution. Comparable to the cell experiments, infusion of the heart with 200 µmol/L H$_2$O$_2$ resulted in an increase in the 405/488 nm ratio, whereas addition of 2 mmol/L dithiothreitol resulted in a decrease (Figure 4B). Mostly, the ventricles are GFP positive in the images, which is in line with the known α-myosin heavy chain promoter activity. Matching the results obtained with the isolated cardiac myocytes, we observed a higher response of the mito hearts toward H$_2$O$_2$ stimulation, which was exemplified by a lower OxD and, thus, E$_{GSH}$ compared with the cyto hearts (Figure 4C).

The Response of the E$_{GSH}$ to Stimulation With Isoprenaline and Angiotensin Differs in the Cytoplasm and Mitochondrial Matrix

On the cellular level, β-adrenergic stimulation-like isoprenaline treatment and angiotensin II stimulation of cardiac myocytes have been described to activate cellular production of reactive oxygen species (ROS).$^{22,23}$ Although there are data indicating that the NADPH oxidase enzymes are involved in the response, it is not clear, if the E$_{GSH}$ after stimulation alters homogeneously in the cytoplasm and the mitochondria. We, therefore, tested the redox response of the cytoplasm and mitochondrial matrix toward β-adrenergic (Figure 5A) or...
angiotensin II stimulation (Figure 5B) for 20 minutes. Most interestingly, the 2 compartments indeed responded differently. Whereas in the cytoplasm, an oxidation response was observed compared with nonstimulated cells, the E_{GSH} in the mitochondrial matrix was only slightly reduced in the mito2 cardiac myocytes. In the mito1 cardiac myocytes, however, no significant difference to nontreated control cardiac myocytes was observed.

Redox Changes in Response to Hypoxia or Ischemia

Previously, it has been shown that oxygen availability affects the cellular redox status. We addressed whether the transgenic cardiac myocytes allow detection of endogenous E_{GSH} changes to hypoxia. To this end, we cultured cyto1 and mito1 cardiac myocytes in 20% O_2 and decreased the oxygen concentration to 1% O_2 (hypoxia) or 0.1% O_2 (severe hypoxia) for 15 minutes followed by reoxygenation to 20% O_2 (Figure 6A and 6B). The onset of hypoxia and severe hypoxia was associated with the reduction of the E_{GSH} in both compartments, which was reversible on reoxygenation. The cytoplasm, however, responded more rapidly to the onset of hypoxia and reoxygenation compared with the mitochondrial matrix.

In contrast to a transient hypoxia, ischemia induces tissue destruction and remodeling in the heart. We induced MI by ligation of the left anterior descending artery. Left anterior descending artery–ligated mice demonstrated a significantly impaired fractional area shortening (Figure 7A) and ejection fraction (Figure 7B) compared with sham-treated mice. Functional impairment was associated with characteristic changes in E_{GSH} in the cytosol and the mitochondrial matrix (Figure 7C). Whereas in the cytosol, the E_{GSH} was unchanged over time comparing MI and sham-treated mice, the mitochondrial matrix was oxidized by roughly 5 mV on days 7 and 14 after MI.

Discussion

Studies of cellular redox alterations have been limited to biochemical assays, and the use of fluorescent chemicals such as dihydrodichlorofluorescein diacetate, dihydrorhodamine, C11-BODIPY, etc. before redox-sensitive biosensors were developed. Fluorescent chemical probes have several disadvantages including lack of ROS specificity, unspecific oxidation, photobleaching, and irreversible reaction with ROS, which makes a dynamic measurement of redox alterations impossible. Genetically encoded reduction–oxidation-sensitive protein probes are mostly YFP or GFP derivatives and were developed to serve as tools for real-time monitoring of the redox potential in living cells and tissues. These biosensors mimic redox relays in which they exchange electrons with oxidoreductases, peroxidases, or other enzymes, which allows specificity and sensitivity of the obtained signals. In this study, we used roGFP2 coupled to the small thiotransferase Grx1, to determine specifically the E_{GSH}. Furthermore, we targeted the biosensor to the cytoplasm or the mitochondrial matrix to characterize redox-active microdomains. To date, just a limited number of mouse models making use of genetically encoded redox biosensors for quantifying the redox potential have been developed. Here, we demonstrate the successful establishment of cardiac transgenic expression of Grx1-roGFP2 for in vitro and in vivo monitoring of the...
glutathione redox potential of isolated cardiac myocytes and the whole heart.

Overall, our data demonstrate that in cardiac myocytes, the cytoplasm and the mitochondrial matrix have unique glutathione redox characteristics under resting conditions and after stimulation. This is in line with a recent report on cytosolic and mitochondrial H$_2$O$_2$ pools analyzed in yeast, in which a newly developed highly sensitive peroxiredoxin-based genetically encoded probe was applied. Cytosolic H$_2$O$_2$ levels were found to be maintained independently from the mitochondrial H$_2$O$_2$ levels. In our study, we found that compared with the cytoplasm, the mitochondrial matrix of cardiac myocytes exhibits a more reduced environment as determined in isolated cardiac myocytes and in Langendorff-perfused hearts. In the literature, it was in contrast for a long time hypothesized that the mitochondrial matrix maintains a relatively oxidizing environment. Mitochondria are the most redox-active compartment of mammalian cells, accounting for more than 90% of electron transfer to O$_2$ as the terminal electron acceptor. Nevertheless, mitochondria are apparently well equipped with reducing defense systems, including coenzyme Q, cytochrome c, superoxide dismutase, catalase, peroxiredoxin, and glutathione peroxidase. Glutathione peroxidase inactivates peroxides using GSH as a source of reducing equivalents. GSH resides in the mitochondria although produced exclusively in the cytosol from its constituent amino acids by the sequential action of γ-glutamylcysteine synthase and GSH synthase. GSH is transported to the mitochondrial matrix by the 2-oxoglutarate carrier and the dicarboxylate carrier. Although the percentage of the total cell GSH content found in mitochondria is minor (ca. 15%), the mitochondrial glutathione concentration is similar to that found in the cytosol. The high ratio of GSH:GSSG in the mitochondria is maintained by the mitochondrial reducing equivalent of NADPH, generated in the Krebs cycle and based on our data is indeed capable of maintaining a high degree of GSH reduction under normal conditions. Maintenance of an appropriate redox balance in mitochondria is of particular importance in the heart, which is an organ that is highly dependent on proper mitochondrial function. Even modest mitochondrial dysfunction is associated with contractile impairment. In general, previous work has shown that there is an inverse relationship between maintenance of redox and energetic balance in the heart, related to the NADH versus NADPH levels. To preserve an optimized proportion between mitochondrial respiration and

![Figure 6. Hypoxia decreases the glutathione redox potential in the mitochondrial matrix and the cytoplasm.](http://circres.ahajournals.org/)

Figure 6. Hypoxia decreases the glutathione redox potential in the mitochondrial matrix and the cytoplasm. Isolated cardiac myocytes derived from the indicated mouse lines were exposed to (A) 1% O$_2$ (hypoxia) or (B) 0.1% O$_2$ (severe hypoxia) for 15 min. Subsequently, cells were reoxygenated by exposing them to 20.9% O$_2$. Ratios of the fluorescence excitations at 405/488 nm were recorded for 25 min every 5 s. For each experiment, at least 40 cardiac myocytes from 4 independent mice were included, mean (black line)±SEM (red lines).
ROS emission, a reduced mitochondrial matrix environment as observed in this study, thus, might be critical.

The heart requires a constant supply of energy to support the contractile activity. This obligation is met by the daily synthesis of ATP via oxidative phosphorylation. During oxidative phosphorylation, electrons are transferred from electron donors to electron acceptors, such as oxygen, in redox reactions. These redox reactions release energy, which is used to form ATP. Oxidative phosphorylation is simultaneously the endogenous source of mitochondrial ROS production and, thus, potentially affects the $E_{GSH}$. We tested how a decline in oxygen availability, that is, hypoxia affects the $E_{GSH}$ in the cytosol and the mitochondrial matrix. Hypoxia (1% O$_2$) and severe hypoxia (0.1% O$_2$) for 15 minutes resulted in a reduced state in the cytoplasm and the mitochondrial matrix, which was almost completely reversible on reoxygenation. Most interestingly, the response was rapid in the cytoplasm, whereas the response in the mitochondrial matrix was time shifted by roughly 2 to 3 minutes demonstrating that in hypoxia, the mitochondria stabilize their $E_{GSH}$ for a longer time before being affected by the hypoxic conditions.

In contrast to an acute and reversible hypoxic event, permanent ischemia as a result of MI and subsequent tissue remodeling processes can induce mitochondrial dysfunction. This can lead to an increased electron leakage from the electron transport chain that, in turn, reacts with residual O$_2$ to give O$_2^{-}$: In line, we found an oxidative alteration of the $E_{GSH}$ mainly in the mitochondrial matrix on days 7 and 14 in cardiac myocytes isolated from mice, which underwent left anterior descending artery ligation compared with sham-treated control mice. In sharp contrast, the cytoplasm did not exhibit a similar oxidation. This indicates that after ischemia the mitochondria are the primary source for the oxidative state most likely because of mitochondrial dysfunction, which is part of the ischemia-induced remodeling process.

Taken together, we have established new biosensor mice to analyze the redox response of cardiac tissue. The glutathione system is one of numerous redox-regulating systems in the heart. It should be highlighted that the applied Grx1-roGFP2 biosensor reports specifically the $E_{GSH}$ in the cytoplasm or the mitochondrial matrix. Using this approach does not take into account the other redox couples or other compartments.
than the cytosol or the mitochondrial matrix. For analyzing, for example, the $E_{GSH}$ in the endoplasmic reticulum roGFP variants such as roGFP-iL, which has a midpoint potential much closer the oxidizing conditions assumed in the ER lumen, need to be applied. At least the $E_{GSH}$ as analyzed in this study responds quickly and dynamically to the changes in the redox status, responds to physiological stimuli such as isoprenaline or angiotensin II, and also reacts to physical interventions such as hypoxia or ischemia. Most interestingly, our data demonstrate that the cytoplasm and the mitochondrial matrix respond separately from each other indicating that it is important to further analyze subcellular redox microdomains to understand the molecular and functional consequences of changes in the redox homeostasis. We anticipate that in this regard the presented mouse models may find useful applications in understanding the redox biology of cardiac myocytes in further depth.

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**Disclosures**

None.

**References**


Novelty and Significance

What Is Known?

• Spatiotemporal regulation of the activity of intracellular proteins and signaling pathways by the subcellular redox environment affects normal cardiovascular function.
• The glutathione redox potential (EGSH) is often used as a measure of the cellular redox environment; however, its precise, dynamic, and specific quantification at subcellular levels with dye compounds is difficult.
• Genetically encoded biosensors such as the Grx1-roGFP2 enable specific analysis of the EGSH in subcellular compartments of living cells.

What New Information Does This Article Contribute?

• Transgenic mice expressing the Grx1-roGFP2 biosensor in the cytoplasm or the mitochondrial matrix of cardiac myocytes allow determining the EGSH in these 2 subcellular compartments without affecting normal heart function.
• The EGSH responds quickly and dynamically to stimuli such as isoprenaline or angiotensin II and is sensitive to physical interventions such as hypoxia or ischemia.
• The cytoplasm and the mitochondrial matrix respond separately from each other with changes in EGSH indicating that subcellular redox microdomains are important for cardiac myocyte function.

Redox changes can stimulate signal–transduction pathways, which are important for cardiac physiology and pathophysiology. Recent developments to quantitatively describe defined redox changes include the application of genetically encoded redox biosensors. We developed α-myosin heavy chain promoter-driven Grx1-roGFP2 transgenic mice, in which the biosensor is either expressed in the cytoplasm or targeted to the mitochondria. Generation of these mice allows quantitative and dynamic measurements of EGSH in the intact cardiac myocytes and in the whole heart. The Grx1-roGFP2 biosensor could be functionally expressed in the mitochondria without impairing the function of the mitochondria or the heart. Quantification of the EGSH in isolated cardiac myocytes and Langendorff-perfused hearts revealed a more oxidized EGSH in the cytoplasm than in the mitochondrial matrix. Distinct subcellular microdomains became also evident after stimulating the cells with isoprenaline and angiotensin II or after exposing them to hypoxia. The described mouse models allow the precise analysis of the EGSH in cardiac myocytes and can be applied to gain insight into the importance of subcellular EGSH compartments for cardiac physiology.
Redox Imaging Using Cardiac Myocyte-Specific Transgenic Biosensor Mice

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Detailed Methods

Cryosections
Animals were sacrificed by cervical dislocation, hearts were removed and mounted supported by a piece of hard-boiled egg white using O.C.T.™ Tissue Tek® (SAKURA) 1. The tissue was frozen using liquid nitrogen and isopentane. 8 µm sections were cut (Microm HM560, Thermo Scientific) and fixed by formaldehyde vapors 2. Sections were counterstained with DAPI and embedded with Fluoromount™ (Sigma Aldrich).

Immunohistochemistry
Paraffin-embedded sections were deparaffinated using xylene, immersed in ethanol and rehydrated. Unmasking of antigens was performed using a microwave and 10 mM boiling citrate buffer. Endogenous peroxidase was quenched in 3% hydrogen peroxide and 0.3% Triton-X in PBS. Nonspecific antibody binding was blocked for 1 h with 10% normal goat serum in PBS. The slides were incubated with anti-GFP-antibody (Cell Signaling, #2956; 1:50) overnight at 4°C. After washing in PBS the slides were incubated for 1 h with biotinylated anti-rabbit antibody (Vector Laboratories, BA-1000, 1:200). Slides were incubated for 1 h in ABC complex (Vector Laboratories, VECTASTAIN ABC HRP Kit), washed with PBS and incubated with DAB substrate (Applichem) for 5 min. The reaction was stopped with water. Mayer´s Hematoxylin solution (Sigma-Aldrich) was used as a nuclear counterstain. Following dehydration with an ethanol series and xylene sections were coverslipped in Roti®-Histol (Roth).

Histological Analysis
For Masson’s Trichrome staining, hearts were fixed in 4% PFA in PBS and embedded in paraffin. Sections were cut at 10 µm.

Fluorescence intensity measurements
Cardiomyocytes and mitochondria were isolated as described in the Methods section. The basal fluorescence was analyzed using 3 µg mitochondria or 750 cells in 500 µL imaging buffer (in mM: NaCl 144, KCl 5.4, MgCl₂ 1, CaCl₂ 1, HEPES 10, pH7.3) by the F-7000 Fluorescence Spectrophotometer (Hitachi). Fluorescence was measured for 30 sec at RT using an excitation wavelength of 405 nm or 488 nm and an emission wavelength of 510 nm using the FL solution software.

Isolation of cardiac fibroblasts
Cardiac fibroblasts were isolated from adult mice. Hearts were excised after cervical dislocation. Hearts were finely minced, and subjected 7 times to repeat digestions at 37°C in Hanks balanced salt solution containing 100 U/ml Collagenase IV and 0.1% Trypsin for 15 min. After each digestion step the tissue was mechanically sheared and the supernatant containing dissociated cells was collected. Cells from the digestions were pooled and re-suspended in DMEM/F-12 Glutamax supplemented with 10% FCS (PAN-Biotech), 1% penicillin/streptomycin and 100 µM ascorbic acid. Cells were plated and incubated to allow for the attachment of fibroblasts. After 2 h the supernatant was replaced by fresh media.

References
Online Figure I: Cardiomyocyte-specific expression of Grx1-roGFP2 in transgenic mice using the αMHC promoter as driver. (A) Confocal microscopy in cryosections of the hearts derived from the indicated mouse lines after staining with DAPI. (B) Cardiac fibroblasts were isolated from hearts of the indicated mouse lines and analyzed by epifluorescence microscopy. As a positive control cardiomyocytes isolated from the heart of a cytosolic sensor mouse line were used. Arrows indicate the non-GFP2 expressing fibroblasts.
Online Figure II: GFP-immunohistochemistry and histology in hearts excised from Grx1-roGFP2 transgenic mice. (A) Immunohistochemistry using anti-GFP antibodies in sections derived from the indicated mouse lines. In the secondary antibody (sec. ab.) control, the experiment was performed with a section from a mito1 mouse without adding the primary anti-GFP antibody. (B) Sections of paraffin embedded hearts after Trichrome staining derived from the indicated mouse lines. (C) cross-sectional and (D) longitudinal areas of the cardiac muscle enlarged from the mouse hearts shown in A.
Online Figure III: Colocalization of roGFP2 with a mitochondrial marker and heart function in Grx1-roGFP2 transgenic mice. (A) Confocal microscopy in isolated cardiomyocytes from the indicated mouse lines after staining with red mitotracker. (B) Isolated cardiomyocytes and (C) isolated mitochondria derived from the indicated mouse lines were analyzed for fluorescence intensity at 405 nm and 488 nm. (D) Intraventricular volume in diastole and systole analyzed by echocardiography of 4-7 mice per genotype and respective wt mice was performed at the age of 8 weeks in the indicated mouse lines. mean ± SEM.
Online Figure IV: Mitochondrial function in Grx1-roGFP2 transgenic mice. (A) Isolated mitochondria from the indicated mouse lines were analyzed for mitochondrial complex I, II, IV and V activity by BN-PAGE analysis. (B) Oxygen consumption rate (OCR) of isolated mitochondria from the indicated mouse lines. n = 9 samples per mouse line.
Online Figure V: Dynamic response of isolated Grx1-roGFP2 cardiomyocytes after subsequent stimulation with \( \text{H}_2\text{O}_2 \) and DTT. Cardiomyocytes isolated from cyto1 and mito1 mice were treated with 100 µM \( \text{H}_2\text{O}_2 \). Subsequently, cells were washed and treated with 1 mM DTT. Ratios of the fluorescence excitations at 405/488 nm were recorded.
Online Figure VI: Response of isolated Grx1-roGFP2 cardiomyocytes derived from the cyto2 and mito2 mouse lines to H$_2$O$_2$ and DTT. Cardiomyocytes isolated from cyto2 and mito2 mice were treated with 100 µM H$_2$O$_2$, 500 µM H$_2$O$_2$, 1 mM DTT or 2 mM DTT. Ratios of the fluorescence excitations at 405/488 nm were recorded. For each concentration at least 45 cardiomyocytes from three (cyto2) and 4 (mito2) independent mice were included. mean ± SEM.
Legends for online videos

Online video I Response of an isolated Grx1-roGFP2 mito1 cardiomyocyte after treatment with DTT. Ratio of the fluorescence excitations at 405/488 nm after a single bolus treatment with 1 mM DTT.

Online video II Response of an isolated Grx1-roGFP2 mito1 cardiomyocyte after treatment with H₂O₂. Ratio of the fluorescence excitations at 405/488 nm after a single bolus treatment with 100 µM H₂O₂.