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**Novel biosensors reveal a shift in the redox paradigm from oxidative to reductive stress in heart disease**

Rhian M Touyz, MD PhD, Aikaterini Anagnostopoulou, PhD, Livia De Lucca Camargo, PhD,  
Augusto C Montezano PhD

Institute of Cardiovascular and Medical Sciences, BHF GCRC, University of Glasgow, United  
Kingdom

**Short title:** Reductive stress and the heart

**Correspondence:**

Rhian M Touyz MBBCh, PhD, FRCP, FRSE.  
Institute of Cardiovascular & Medical Sciences  
BHF Glasgow Cardiovascular Research Centre  
University of Glasgow  
126 University Place  
Glasgow G12 8TA  
Tel: + 44 (0)141 330 7775/7774  
Fax: + 44 (0)141 330-3360  
Email: [Rhian.Touyz@glasgow.ac.uk](mailto:Rhian.Touyz@glasgow.ac.uk)

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Maintaining intracellular redox balance through tightly controlled systems that regulate production (pro-oxidants) and scavenging (anti-oxidants) of reactive oxygen species (ROS) is critical for normal cell function. In the heart, ROS are produced primarily by mitochondria as a byproduct of electron transport and also by extra-mitochondrial enzymes, such as NADPH oxidases (Nox). Multiple antioxidants including superoxide dismutase, catalase, glutathione peroxidase and the thioredoxin/thioredoxin-reductase systems counterbalance ROS generation, preserving redox equilibrium. Disruption in this equilibrium leads to redox stress, of which two main types have been defined: oxidative stress and reductive stress. Oxidative stress, characterised by a shift in the oxidative:reductive potential to a more oxidative state due to increased ROS production by pro-oxidant enzymes and/or reduced anti-oxidant defence mechanisms to scavenge excessive ROS, plays a physiological role in aging and wound healing, and a pathophysiological role in atherosclerosis, hypertension, heart failure and ischemia-reperfusion injury (1). Reductive stress, on the other hand, is characterised by an aberrant increase in reducing equivalents, such as reduced glutathione (GSH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH), increased activation of antioxidant enzymes and reduced pro-oxidant capacity, leading to a shift in the redox balance from an oxidative to a reduced state (2). Intuitively one would consider activation of antioxidant systems as being redox-protective, however, growing evidence indicates that reductive stress may be even more injurious than oxidative stress through processes whereby intrinsic cytoprotective defences negatively target the exact systems they should protect (3). In the heart, reductive stress has been linked to mitochondrial dysfunction, heart failure, myocardial ischemia-reperfusion injury, cardiac hypertrophy and cardiomyopathy. These are the exact pathological conditions associated with oxidative stress. How then can both an increase in pro-oxidants and an increase in anti-oxidants or reductants lead to the same outcome?

In the current issue, Swain et al shed some light on this enigma (4). A novel cardiomyocyte-specific glutaredoxin 1-reduction-oxidation sensitive (ro) green fluorescent probe (GFP) (Grx1-roGFP2) was employed to create a unique mouse model where the glutathione redox potential can be measured (4). Although redox biosensors have been utilised extensively to label and track reactive oxygen species in cell-based systems (5), the novelty of the GRX1-roGFP2 mouse model is that it allows for assessment of the redox potential in cardiomyocytes in a dynamic and quantitative manner specifically in the mitochondrial and cytoplasmic compartments, in the intact mouse. Advantages of the biosensor approach compared with the frequently used fluorescence probe-based strategy relates to greater oxidant species specificity and sensitivity, lack of photobleaching and facility to track reactive oxygen species spatially.

The powerful biosensor cardiac transgenic model described in the current issue of the journal (4) revealed distinct glutathione redox microdomains in the mitochondria and cytoplasm of cardiomyocytes. In particular these compartments have unique glutathione redox features, which change in basal and stressed conditions. Of major significance and rather surprisingly, the mitochondrial matrix of cardiomyocytes exhibit a highly reduced redox potential compared with the cytoplasm (4) posing the question why mitochondria would maintain a state of reductive stress relative to oxidative stress in the cytoplasm? This may relate to the fact that mitochondria are the most redox-active compartment in cells. They possess a rich network of reducing defence systems including glutathione peroxidase, which inactivates  $H_2O_2$  using GSH as a reducing equivalent (6). While GSH is produced in the cytoplasm it accumulates in the mitochondrial matrix, where it exists primarily in the reduced state, in large part by NADPH produced in the Krebs cycle (7). There is thus important cross-talk between the cytoplasmic and mitochondrial matrices, which impacts redox state globally and in a compartment-specific manner. Maintaining mitochondrial energetic balance and the reductive redox state are critical for normal mitochondrial function (8). This is especially relevant in the heart, which requires a continual supply of energy through oxidation-phosphorylation-regulated ATP production in mitochondria.

Mitochondrial redox stress is finely tuned and even small changes in the oxido-reductive profile promotes mitochondrial dysfunction and impaired cardiac contraction (9). This is highlighted in isolated cardiomyocytes from GRX1-roGFP2 mice which when challenged with acute stresses that

increase ROS generation (isoprenaline, angiotensin II, hypoxia) exhibit reductive stress in both the cytoplasm and the mitochondria, processes that are reversed with reoxygenation (4). On the other hand cardiomyocyte exposure to prolonged stimulation resulted in a shift towards a more oxidised milieu in the cytoplasm, phenomena also observed in cardiac mitochondria in GRX1-roGFP2 mice exposed to chronic cardiac ischemia induced by left anterior descending artery (LAD)-ligation. This change in mitochondrial stress from a reductive to an oxidative state likely contributes to myocardial remodelling and injury following an ischemic event (4). Together these findings highlight the dynamic and labile state of redox stress in the heart, determined in large part by ROS compartmentalisation, antioxidant bioavailability and the nature of the stimulus. The physical and functional compartmentalization of oxido-reductive phenomena in mitochondria versus cytoplasm likely plays an essential role in redox signaling. Localization of ROS in discrete microdomains and redoxomes (redox-active endosomes/organelles) where oxidants are produced and where they interact with their specific molecular targets is critical for signaling specificity (10).

The concept of reductive stress and cell function is not new. Already in the 1980s, it was shown that chemical hypoxia induces hepatocyte swelling, bleb formation, and loss of cell viability through reductive stress caused by respiratory inhibition and formation of 'toxic' oxygen species (11). Increased reductants have also been implicated in reductive repair of protein radicals in an oxidative stress environment. In a transgenic mouse model of human cardiomyopathy caused by a mutation in the gene encoding  $\alpha$ B-crystallin (mutant protein aggregation cardiomyopathy), protein accumulation was associated with an increase in the generation of GSH and NADPH, increased activity of antioxidant enzymes and reductive stress (12). This seminal study was amongst the first to demonstrate a distinct role for reductive stress in human cardiac disease (12). More recently, this notion has been extended to vascular disease in humans where loss of function of glutathione peroxidase-1 (GPX1) was found to induce both oxidative and reductive stress, with reductive stress driving S-glutathionylation of ROS1 tyrosine phosphatase SHP-2 and processes leading to vascular remodelling (13). These findings, together with those highlighted in the current issue (4), suggest that it is timely to rethink the current dogma that oxidative stress is the major redox contributor to pathological processes underlying cardiovascular disease. Despite the extensive experimental and clinical evidence demonstrating hyperactivation of ROS-generating oxidases, increased ROS levels, reduced antioxidant capacity and increased oxidative stress, we now need to include in the redox paradigm of disease, the importance of antioxidant systems that facilitate increased generation of reducing equivalents and consequent increased reductive stress. This change in concept will demand a greater understanding of the processes that cause a shift of oxido-redox stress towards a reductive state and will require new robust models and approaches.

Although the mechanisms that cause and regulate reductive stress are elusive, a number of factors have been implicated including activation of nuclear erythroid-2 like factor-2 (Nrf2), heat shock protein (HSP), reactive oxygen species and glucose 6 phosphate dehydrogenase (G6PD) (14). In addition regulation of ROS scavenging by antioxidants in a compartment-specific manner influences redox status. Using targeted viral gene transfer vectors expressing redox-sensitive GFP fused to sensor domains to measure oxidized glutathione or  $H_2O_2$  in cultured H9c2 cardiomyocytes, it was found that antioxidant flux is dependent on mitochondrial substrate catabolism and the mitochondrial thioredoxin- and glutathione-driven antioxidant network (15). The H9c2 model also revealed that ROS scavenging by mitochondrial antioxidants influences ROS regulation in the cytoplasmic compartment, similar to what was revealed in the cardiac myocyte-specific Grx1-roGFP2 sensor mice (4), thus supporting the notion of redox interplay between mitochondrial and cytoplasmic subcellular compartments.

While the field of reductive stress is still immature, the novel findings reported in the current issue of the journal (4) highlight the importance of both extremes of redox balance in ischaemic cardiac injury with clear evidence that oxidative and reductive stress in a subcellular compartment-specific manner play a role in redox control of the heart. What still remains unclear however is whether the damage induced by both forms of redox stress is oxidative in character. Unfortunately in the study under discussion, this was not interrogated with reactive oxygen species scavengers, such as

N-acetyl cysteine, tempol or tiron, Another drawback of the study is that the glutathione redox potential was only examined in the mitochondrial and cytoplasmic domains of cardiomyocytes, ignoring the potentially important contribution of oxido-reductive stress in other organelles such as the endoplasmic reticulum, especially in relation to the unfolded protein response (endoplasmic reticular stress) associated with cardiac injury. Despite these limitations, Swain et al (4) have advanced the field, not only by adding a new biosensor model to the toolbox of redox probes, but by demonstrating that there is a complex cooperative redox network that impacts redox stress in cardiac health and disease. Reductive stress appears to be as important as oxidative stress in ischemic cardiac injury. As the field matures, the exact interplay between oxido-reductive stress will become more evident.

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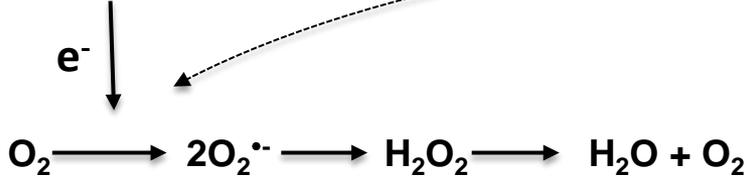
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## **Figure legend**

Schematic demonstrating the interplay between processes that cause oxidative and reductive stress in cardiomyocytes. Cardiac stressors, such as ischemia, hypoxia, reactive oxygen species (ROS), vasoactive agents, induces ROS generation through mitochondrial and NADPH oxidase sources. In addition, these stimuli activate mitochondrial anti-oxidant systems and increased generation of reducing equivalents. Redox processes are tightly regulated in subcellular compartments, including the mitochondria and cytoplasm where ROS influence distinct signaling pathways. These processes may be modulated by the state of oxido-reductive stress. The role of endoplasmic reticular (ER) stress is unclear in this paradigm. Ang II, angiotensin II; GSH, reduced glutathione; GSSG, glutathione disulfide; GPX, glutathione peroxidase; GSR, Glutathione reductase; G6PD, glucose-6-phosphate dehydrogenase; SOD, superoxide dismutase.

**Cell Stressor**  
Ischemia  
Hypoxia  
ROS, Ang II

**NADPH Oxidase**

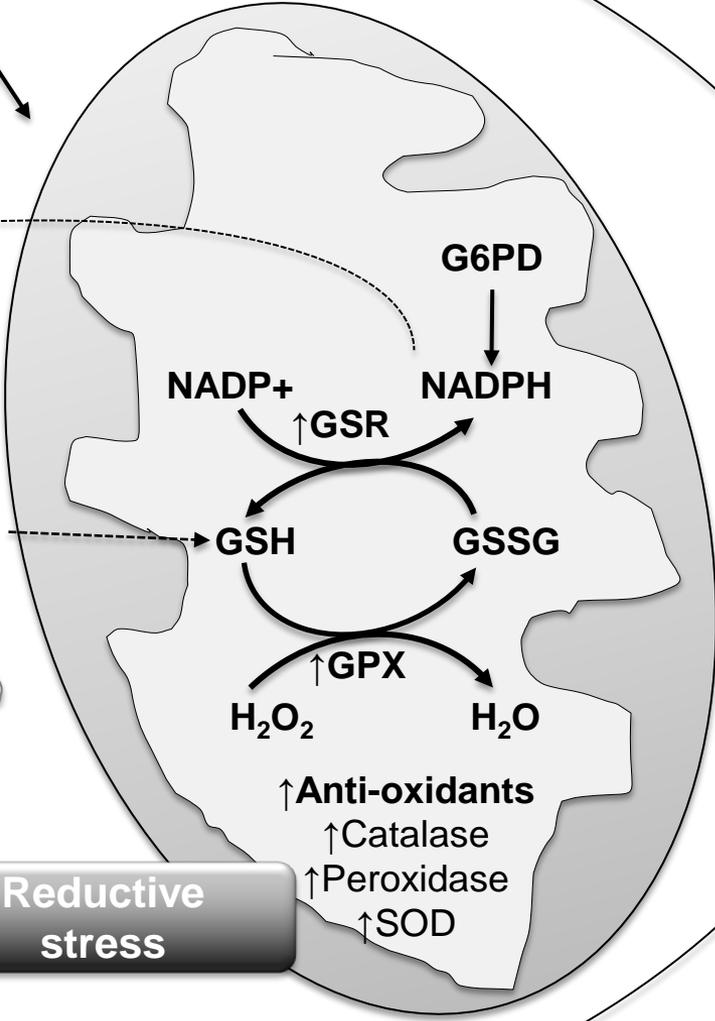


**↑ROS**

ER Stress

**Oxidative stress**

GSH



**Reductive stress**

**Redox signaling** → **Cardiac injury**