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Redox signalling occurs when a biological system alters in response to a change in the level of a particular reactive oxygen species (ROS) or the shift in redox state of a responsive group such as a dithiol–disulphide couple (D’Autreaux and Toledano, 2007; Finkel, 2011; Fourquet et al., 2008; Janssen-Heininger et al., 2008; Rhee, 2006). Although ROS are best known as damaging agents in pathology, a more nuanced view has developed. It is now clear that some ROS, such as hydrogen peroxide (H₂O₂), can act as messengers both in the extracellular environment and within cells (D’Autreaux and Toledano, 2007; Fourquet et al., 2008; Janssen-Heininger et al., 2008; Rhee, 2006). Mitochondria seem to be an important redox signalling node, partly because of the flux of the ROS superoxide (O₂⁻) generated by the respiratory chain and other core metabolic machineries within mitochondria (Balaban et al., 2005; Finkel, 2011; Murphy, 2009a). In addition, the mitochondrial matrix is central to metabolism, as oxidative phosphorylation, the citric acid cycle, fatty acid oxidation, the urea cycle and the biosynthesis of iron sulphur centres and haem take place there. Furthermore, mitochondria have key roles in apoptosis, calcium homeostasis and oxygen sensing (Duchen, 2004; Murphy, 2009a; Murphy, 2009b). Consequently, mitochondria are at the core of many biological processes, and redox signals to and from this organelle help to integrate mitochondrial function with that of the cell and organism. In this Cell Science at a Glance article we outline how mitochondrial redox signals are produced and modulated, the mechanisms by which redox signals can alter mitochondrial function and the experimental procedures available to assess this.

Production and modulation of redox signals to and from mitochondria

The initial ROS formed within mitochondria is O₂⁻, which is generated by the respiratory chain and other enzymatic components within the mitochondrion (Finkel, 2011; Murphy, 2009a).
Mitochondrial $O_2^-$ generation provides an indication of functional status because its production is altered by many cellular factors. These include the membrane potential, the reduction state of electron carriers and post-translational modification or damage to the respiratory chain (Murphy, 2009a). However, $O_2^-$ itself is not the main ROS signal within mitochondria because it is mostly converted to $H_2O_2$ by manganese superoxide dismutase (MnSOD), which reacts very rapidly with $O_2^-$ and is present at a high concentration within the matrix (Balaban et al., 2005; Chance et al., 1979; Finkel, 2005; Murphy, 2009a). As $H_2O_2$ can pass easily through mitochondrial membranes, it can act as a redox signal from mitochondria to the rest of the cell and vice versa (Balaban et al., 2005; D’Autreaux and Toledano, 2007; Droge, 2002; Fourquet et al., 2008; Janssen-Heininger et al., 2008; Murphy, 2009a).

Respiratory complex III can also release $O_2^-$ into the intermembrane space (St-Pierre et al., 2002; Muller et al., 2004; Han et al., 2001). The intermembrane space enzyme p66Shc (the 66 kDa isoform of the growth factor adapter Shc) can also generate $O_2^-$, which can regulate apoptotic cell death (Giorgio et al., 2005). The $O_2^-$ can diffuse from the intermembrane space to the cytosol or be converted to $H_2O_2$ by an intermembrane space Cu,Zn-SOD (Okado-Matsumoto and Fridovich, 2001). The Mia40p and Ets1p system of the intermembrane space, which inserts disulphide bonds into intermembrane space proteins during import, also generates $H_2O_2$ (Koehler et al., 2006), but the potential of this for redox signalling is unclear.

Matrix $H_2O_2$ concentration is further regulated by degradation through peroxiredoxin 3 and 5 (Prx3 and Prx5, respectively) and glutathione peroxidase 1 (Gpx1), with Prx3 being the most significant because of its relative abundance and reactivity (Cox et al., 2010). Prx proteins degrade $H_2O_2$ using the mitochondrial thioredoxin 2 (Trx2) system as a reducing source, whereas Gpx1 uses the mitochondrial glutathione (GSH) pool (Cox et al., 2010). During its reaction cycle, dimeric Prx3 forms an inter-subunit disulphide that is reduced back to the dithiol form by Trx2 (Rhee, 2006; Rhee et al., 2001). Exposure to $H_2O_2$ can lead to a significant fraction of Prx3 being in the disulphide form at any given time, thereby affecting $H_2O_2$ release from mitochondria (Cox et al., 2009; Cox et al., 2008). The activity of Prx3 might also be affected by post-translational modification or by the extent of its oligomerisation (Rhee et al., 2001; Rhee et al., 2005b; Cox et al., 2010). The extent of this $H_2O_2$ signal can be modulated both by its production, which is highly responsive to mitochondrial status (Murphy, 2009a), and by the rate of its degradation by matrix peroxidases – predominantly Prx3 – and diffusion into and out of the organelle.

The $H_2O_2$ that is produced by one mitochondrion can diffuse to another, coordinating or relaying signals between the organelles (Murphy, 2009a). Additionally, $H_2O_2$ can diffuse to mitochondria from the cell surface through the activation of NADPH oxidase (NOX) enzymes by growth factors (Janssen-Heininger et al., 2008; Rhee et al., 2005a; Rhee et al. 2005b).

The main ROS involved in redox signalling to and from mitochondria seems to be $H_2O_2$; however, other forms of ROS can also contribute. Nitric oxide (NO) is generated by NO synthases, and can diffuse into mitochondria and modulate mitochondrial function by competing with $O_2$ at respiratory complex IV – thereby slowing respiration – and by the $S$-nitrosation of mitochondrial thiol groups (Moncada and Erusalimsky, 2002). Iron sulphur centres in proteins such as aconitase can react rapidly with $O_2^-$ (D’Autreaux and Toledano, 2007), thereby modifying activity independently of $H_2O_2$. In addition, $O_2^-$ can diffuse from the intermembrane space through the outer membrane voltage-dependent anion channel to the cytosol, where it can act as a redox signal (Zhou et al., 2010). However, as $O_2^-$ is shorter lived and less diffusible than $H_2O_2$, its signalling roles are thought to be more limited. A number of other redox signals might also be produced within mitochondria, including peroxynitrite (ONOO–) and the products of mitochondrial lipid peroxidation, such as prostaglandin-like molecules and 4-hydroxyxynonenal (HNE) (Levonen et al., 2004). These compounds can modify mitochondrial protein thioles and, thereby, affect their activity; however, the metabolic significance of these interactions is unclear.

**Post-translational protein modification by $H_2O_2$ and NO**

To act as effective biological messengers, molecules such as $H_2O_2$ and NO have to bring about a reversible change in the activity of a protein. Generally, this involves modification of a thiol group on a cysteine residue that mediates redox signalling (Eatont 2006; Gilbert, 1990; Gilbert, 1995; Schafer and Buettner, 2001). For example, when $H_2O_2$ acts as a redox signal it oxidises the thiol group on the target protein to a disulphide group, thereby changing the function of the protein; once the level of $H_2O_2$ has returned to basal levels the alteration is reversed and the activity of the protein reverts to its initial level (Beltran et al., 2000; D’Autreaux and Toledano, 2007; Hess et al., 2001; Jacob et al., 2003; Janssen-Heininger et al., 2008; Ziegler, 1985). If the modification is to an active-site thiol, for example oxidation of the crucial thiol in tyrosine phosphatases (Boivin et al., 2010), then the impact on the protein is a clear loss of function. However, thiol oxidation can alter proteins and, thereby, mediate the redox signal in other ways, such as by changing binding affinity to another protein, altering its action as a transcription factor, or by modifying the activity of a transporter or channel (Balaban et al., 2005; D’Autreaux and Toledano, 2007; Droge, 2002; Fourquet et al., 2008; Murphy, 2009a; Rhee, 2006; Rhee et al., 2000).

Generally, in response to $H_2O_2$, protein thiol groups will initially form a sulphenic acid (–SOH) (Brennan et al., 2004; Charles et al., 2007; Cotgreave and Gerdes, 1998; Fratelli et al., 2004; Leonard et al., 2009; Seres et al., 1996; Ziegler, 1985; Dalle-Donne et al., 2008; Dalle-Donne et al., 2009), which can occur by direct reaction of $H_2O_2$ with the thiolate (–S–). This reaction is dependent on the local environment of the thiol and also its pKa, which can lead to certain thiols being particularly sensitive to oxidation. Once formed, the sulphenic acid can itself be a relevant post-translational modification, or it can form other post-translational modifications by reacting with a GSH to form a glutathionylated protein, with an adjacent thiol to form a disulphide (Brennan et al., 2004; Charles et al., 2007; Dalle-Donne et al., 2009; Delaunay et al., 2002; Hurd et al., 2008), or with amides within the protein to form a sulphenyl amide (Sivaramakrishnan et al., 2010). An alternative route to thiol oxidation during redox signalling is the single-electron oxidation of a thiol to a thiyl radical (–·S), which can then react to form disulphide bonds with GSH or with another protein thiol (Woodman and Von Sonntag, 1995; Winterbourn, 1993).

NO metabolism can also modify a protein thiol group into an $S$-nitrosothiol group (SNO) in a process known as $S$-nitrosation or $S$-nitrosylation (Beltran et al., 2000; Hess et al., 2001; Hogg, 2002; Stamler, 1994; Stamler and Hausladen, 1998). The mechanism of SNO formation in vivo is obscure (Hogg, 2002) but, once generated, the SNO can be passed between thiols by transnitrosation, with the formation and stability of the SNO determined by protein sequence motifs that surround the modified cysteine residue (Benhar et al., 2009; Doulias et al., 2010; Hou et al., 1996; Marino and Gladyshev, 2010; Nikitovic and Holmgren, 1996). In addition, an initial SNO on a protein can be modified into other thiol-based groups, such as disulphide, sulphenic acid or into a glutathionylated protein (Nikitovic and Holmgren, 1996; Stamler et al., 1992).
Biologically important mitochondrial redox signals

The concept of redox signalling in biology initially emerged from studies on ROS production from NOXs and on the interactions of NO with biological systems (reviewed by, Finkel, 2011; Rhee, 2006; Janssen-Heininger et al., 2008). Since then, mitochondria have emerged as an important node of redox signalling in numerous biologically important areas. Among the most intriguing is the role of mitochondrial ROS in O2 sensing, especially during hypoxia (Guzy and Schumacker, 2006; Guzy et al., 2008; Patten et al., 2010; Brunelle et al., 2005). In this process, it seems that the production of O2·− by the respiratory chain increases under conditions of low O2 levels (Chandel et al., 1998; Chandel et al., 2000; Guzy et al., 2005). The site of the O2·− production is thought to be respiratory complex III, but the mechanism is unclear (Chandel et al., 2000; Guzy et al., 2005). The elevated mitochondrial O2·− is converted to H2O2 in the mitochondrial matrix, followed by diffusion into the cytosol where it stabilises hypoxia-inducible factor-1α (HIF-1α), thus leading to the transcription of genes that enable the cell to respond to hypoxia (Sanjuán-Pla et al., 2005). Redox signalling by mitochondrial ROS is now implicated in a disparate range of biologically important areas, including as a determinant of chronological lifespan (the time cells in a stationary phase culture remain viable) in yeast (Bonawitz et al., 2007; Pan et al., 2011; Bell et al., 2007), a factor controlling lifespan in Caenorhabditis elegans (Lee et al., 2010; Yang and Hekimi, 2010; Schulz et al., 2007; Hekimi et al., 2011), in the regulation of the immune system (West et al., 2011; Zhou et al., 2011; Wang et al., 2010), in angiotensin II signalling (Dai et al., 2011), in insulin secretion (Leloup et al., 2009) and mitochondrial homeostasis (St-Pierre et al., 2006).

How to investigate redox signalling pathways

Although there is considerable evidence indicating the importance of mitochondrial redox signalling, changes in ROS concentration or a thiol modification also occur during pathologies. Consequently, it is imperative not to assume that such events are necessarily evidence of a redox signal, and to show that changes in the levels of a particular ROS and the subsequent modification of target proteins correlate with and are sufficient to explain the biological modification. However, assessing changes in ROS and protein redox modifications in biological systems is technically demanding and requires an understanding of the underlying chemistry (Murphy et al., 2011). Despite this, considerable evidence demonstrates the presence of protein thiols within mitochondria that can be modified by H2O2 and S-nitrosating agents (Chouchani et al., 2010; Hurd et al., 2005a; Hurd et al., 2005b; Hurd et al., 2007; Prime et al., 2009; Sun et al., 2007). There are now a variety of methods that can be used to assess the levels of particular ROS within mitochondria, and these include mitochondria-targeted small-molecule fluorescence probes (Dickinson et al., 2010a; Dickinson et al., 2010b; Robinson et al., 2006), the use of mitochondria-targeted proteins derived from green fluorescent protein – whose fluorescence is redox sensitive (Meyer and Dick, 2010), and mitochondria-targeted mass spectrometry probes that enable mitochondrial ROS levels to be estimated in vivo (Cochemé et al., 2011). The proteins modified and the nature of the thiol modification can also be determined by using a number of redox proteomic techniques (Chouchani et al., 2010; Dahm et al., 2006; Danielson et al., 2011; Taylor et al., 2003; Held et al., 2010; Hurd et al., 2007).

Once the involved cysteine residues have been determined it is vital to quantify the extent of the modification to ensure that it correlates with a change in protein activity that is sufficient to account for the phenotypic change (Murphy et al., 2011). Mass spectrometric techniques to assess this are now available (Danielson et al., 2011; Held et al., 2010). Proteomic approaches have also been extended to in-vivo models and a range of mitochondrial proteins have been identified that have reversible modifications (Burwell et al., 2006; Doulias et al., 2010; Charles et al., 2007; Fratelli et al., 2003; Murray et al., 2011; Schroder and Eaton, 2008; Sun and Murphy, 2010; Nadtochiy et al., 2007). Without such measurements it might be that the changes in the level of the putative signalling ROS and in the protein redox modification merely correlate with the change in activity, rather than cause it.
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