Review Article

Antioxidant responses and cellular adjustments to oxidative stress

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Abstract

Redox biological reactions are now accepted to bear the Janus faceted feature of promoting both physiological signaling responses and pathophysiological cues. Endogenous antioxidant molecules participate in both scenarios. This review focuses on the role of crucial cellular nucleophiles, such as glutathione, and their capacity to interact with oxidants and to establish networks with other critical enzymes such as peroxiredoxins. We discuss the importance of the Nrf2-Keap1 pathway as an example of a transcriptional antioxidant response and we summarize transcriptional routes related to redox activation. As examples of pathophysiological cellular and tissular settings where antioxidant responses are major players we highlight endoplasmic reticulum stress and ischemia reperfusion. Topologically confined redox-mediated post-translational modifications of thiols are considered important molecular mechanisms mediating many antioxidant responses, whereas redox-sensitive microRNAs have emerged as key players in the posttranscriptional regulation of redox-mediated gene expression. Understanding such mechanisms may provide the basis for antioxidant-based therapeutic interventions in redox-related diseases.

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Contents

1. Introduction ......................................................... 184
2. Glutathione and glutathione-related post-translational modifications ......................................................... 184
2.1. GSH-related enzymatic antioxidants. ......................... 185
3. Redox-dependent transcriptional and post-transcriptional regulation of antioxidants: main examples. ..................... 186
3.1. The Nrf2-Keap1 pathway ..................................... 186
3.2. NF-κB ............................................................. 187
3.3. AP-1 ............................................................. 187
3.4. Role of microRNAs in antioxidant responses ............... 188
4. Antioxidant responses in specific pathophysiological contexts ................................................................. 189
4.1. ER stress ....................................................... 189
4.2. Ischemia–reperfusion ........................................ 190
4.2.1. Post-translational modifications of mitochondrial proteins in ischemia–reperfusion. ........................ 190
4.2.2. The Nrf2 pathway in ischemia–reperfusion .............. 191
4.2.3 Lipid peroxidation products in cardioprotection against ischemia–reperfusion ............................. 191
5. Signal transduction in the context of antioxidant responses: some key players ................................................. 191
5.1. Cytosolic regulation .......................................... 191
5.1.1. Protein tyrosine phosphatases .......................... 191
5.1.2. Protein Kinases .............................................. 192
5.2. Mitochondrial regulation ...................................... 193
5.2.1. Targeting mitochondria to eliminate ROS production ............................................................. 193

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1. Introduction

Free radicals and oxidant species may behave as deleterious and toxic products, involved in cellular and tissular dysfunction. Overproduction of these species may result in DNA, lipid and protein damage. Nevertheless, low or moderate concentrations of reactive oxygen species (ROS) or reactive nitrogen species (RNS) are also involved in physiological responses as part of signal processing and defense mechanisms against infectious agents. In fact, ROS protect the cell against ROS damage by inducing different antioxidant responses and re-establishing or maintaining redox homeostasis. For the sake of clarity in this review we will focus on endogenous antioxidant systems and the different factors involved in their regulation.

2. Glutathione and glutathione-related post-translational modifications

Antioxidant molecules are in fact nucleophilic and reductant molecules able to react with oxidants, which are generally electrophiles, giving them one or two electrons. Glutathione (GSH) is considered the most abundant molecule among endogenous antioxidants. GSH is a reduced peptide consisting of three-residues (γ-glutamyl-cysteinyl glycine) which can donate an electron with the consequence that two electron donating GSH molecules form oxidized GSSG. In humans, GSH is almost uniquely present in a quite high concentration (1–10 mM) which allows to scavenge ROS either directly or indirectly [1]. It can directly react with O$_2^-$ and some other ROS, but its indirect ROS-scavenging functions, such as revitalizing other antioxidants, are likely more important; e.g., it can reduce dehydroascorbic acid which is formed in the reconversion of α-tocopheroxyl radical to α-tocopherol, a lipophilic chain breaking antioxidant, which interacts with the polyunsaturated acyl groups of lipids, stabilizes membranes and scavenges various reactive oxygen species and lipid oxy-radicals [2,3].

As an antioxidant it reacts with ROS, RNS and radicals produced in association with electron transport, xenobiotic metabolism and inflammatory responses [4,5]. GSH is synthesized from its constituent amino acids forming a tripeptide thiol and this synthesis requires two ATP-dependent steps (Fig. 1). The first and limiting step is catalyzed by γ-glutamyl-cysteine ligase (GCL) and the second step mediated by GSH synthetase (GS). GCL is a heterodimeric enzyme composed by a heavy subunit GCLc (73 kDa) with catalytic activity and a smaller one, GCLm (33 kDa) that has a regulatory role on the other subunit [6]. GSH homeostasis in the cell is not only regulated by its de novo synthesis, but also by other factors such as utilization, recycling and cellular export. This redox cycle is known as the GSH cycle and incorporates other important antioxidant, redox-related enzymes. Aerobic respiration may result in an increase in hydrogen peroxide, which will be metabolized by glutathione peroxidase (GPx) by converting 2 GSH molecules to its oxidized form (GSSG). GSH is recycled by the action of glutathione reductase (GR, see below). Moreover, GSH is also able to react with critical Cys residues in proteins, by forming mixed disulfides (see below). This is a labile process that can be reverted by glutathione S-transferase (GST). Another cellular mechanism used to prevent redox unbalance is exporting GSSG to the extracellular medium. In addition, the biosynthetic capacity to form GSH is also regulated by allosteric mechanisms and substrate abundance. An excess of GSH in the cell produces a competitive inhibition in GCLc, while i-Cys is one of the limiting substrates [6,7].

Alterations in the ratio of the redox pair 2GSH/GSSG towards a more oxidized status form the biochemical basis of targeting redox-sensitive cysteine residues in proteins by generating mixed disulfides between the thiol and GSH [2,8]. However, the calculation and quantification of this ratio is not easy and depends on the detection method, the tissue of interest and the abundance of recycling enzymes such as GPx1 [9]. This post-translational modification, known as S-glutathionylation, is a reversible process, which has been extensively reviewed elsewhere [10,11]. S-glutathionylation may result in the activation or inactivation of protein function [12]. Thereby S-glutathionylation is able to modulate different cellular pathways, and affect gene expression profiles by affecting different transcription factors as Nrf2 (see below), or NF-κB.

Although these mechanisms were initially proposed to be part of a protective pathway against other irreversible oxidative modifications [2,8], it was recently shown that S-glutathionylation of eNOS at Cys689 and Cys908 leads to eNOS uncoupling, diminished NO production, and enhanced oxidative stress and superoxide anion production [13]. Thus, S-glutathionylation may be a double-edged sword in the sense that it may promote antioxidant or pro-oxidant responses.
2.1. GSH-related enzymatic antioxidants

Glutathione reductase (GR) is the enzyme critical for the reduction of GSSG back to GSH. GR utilizes FAD and NADPH to reduce one GSSG to two GSH (Fig. 2). The link of GR to oxidative stress was established due to its role in the ROS-releasing macrophage oxidative burst response as cells lacking GR had a more persistent response. In addition, GR deficiency has been linked to lupus erythematosus in Afro-Americans, favism and cataracts [14]. Glutathione peroxidases (GPXs; GPX1–8) couple oxidation of GSH with detoxification of H₂O₂. GPX1 deficiency in mice was also shown to contribute to the development of cataracts at an early age. From the GPX family members known, GPX1 is the ubiquitous enzyme that reduces cytosolic mitochondrial and in some cells peroxisomal peroxides. GPX2 is an epithelial-specific enzyme that reduces disulfide (S–S) bonds in proteins (Prx), and glutathione S-transferase (GST) is using GSH to conjugate and thus to detoxify reactive electrophilic compounds (R).

![Fig. 2. Interconnection of glutaredoxin, peroxiredoxin, thioredoxin, and glutathione containing antioxidant systems. Hydrogen peroxide (H₂O₂) can be reduced by peroxiredoxins (Prx) or glutathione peroxidases (GPX), which couple reduction of H₂O₂ with oxidation of glutathione (GSH). Oxidized Prx can be reduced by thioredoxin (Trx). Subsequently, oxidized Trx become reduced by thioredoxin reductase (TrxR) in a NADPH-dependent manner. Oxidized glutathione disulfide (GSSG) is reduced by glutathione reductase (GR) in the presence of NADPH. Further, glutaredoxins (Grx) can reduce disulfide (S–S) bonds in proteins (Prx), and glutathione S transferase (GST) is using GSH to conjugate and thus to detoxify reactive electrophilic compounds (R).](image)

![Fig. 3. Two-step mechanism reaction of a peroxiredoxin dimer with hydrogen peroxide. (i) the sulfhydryl group at the peroxidatic cysteine of one peroxiredoxin (Prx) subunit is oxidized to sulfenic acid (SOH); (ii) the sulfenic acid condenses with the –SH group at the resolving cysteine from the other subunit to form an intersubunit disulfide bond. The disulfide bond can be reduced by thioredoxin or another reductase (red) depending on the species. Continuous peroxide signaling leads to irreversible hyperoxidation and formation of sulfonic acid (SOOH) at the peroxidatic cysteine to (shown as the “Hyperoxidation”). The species peroxynitrite (ONOO–) has been included in the cartoon because it is susceptible of reduction by peroxiredoxins.](image)
cytosol and peroxisomes. In contrast, a Mn-dependent MnSOD (SOD2) is found in the mitochondrial matrix. A third extracellular variant, EcSOD or SOD3, is secreted and forms a glycosylated heterotetramer bound to the extracellular matrix [35] but again the enzyme activity is Cu-, Zn-dependent. Importantly, changes in the cytosolic Cu/ZnSOD (SOD1), mitochondrial MnSOD (SOD2) or extracellular EcSOD (SOD3) activity or expression correlate with ROS levels [36].

Dysfunction or failure in the dismutation process is linked to the pathogenesis of a number of diseases [36]. Thereby the impact and tissue affected by the different SODs seems to vary; SOD1 has been clearly linked to amyotrophic lateral sclerosis, the extracellular superoxide dismutase (SOD3, ecSOD) seems to be involved in hypertension, acute respiratory distress syndrome, or chronic obstructive pulmonary disease [37] and SOD2 has been linked with carcinogenesis [36]. The removal of superoxide anion by superoxide dismutase(s) appears to be important for a number of cellular processes, among them mitochondrial and ER function (see below).

3. Redox-dependent transcriptional and post-transcriptional regulation of antioxidants: main examples

Multiple H2O2 sensors and pathways are triggered converging in the regulation of transcription factors, including AP-1, Nrf2, CREB, HSF1, HIF-1, TP53, NF-xB, Notch, SP1 and CREB-1, which induce the expression of a number of genes, including those required for the detoxification of oxidizing molecules and for the repair and maintenance of cellular homeostasis, controlling multiple cellular functions like cell proliferation, differentiation and apoptosis. In addition, the family of FoxO-related transcription factors plays an important role in redox responses and is the object of a separate review within this thematic series. H2O2-mediated regulation of transcription factors can take place at different levels: synthesis/degradation, cytoplasm-nuclear trafficking, DNA binding and transactivation [31]. In this section we focus on the most important transcription-related pathways involved in antioxidant responses.

3.1. The Nrf2-Keap1 pathway

The Nrf2 (nuclear factor erythroid 2 [NF-E2]-related factor 2)-Keap1 (Kelch-like ECH-associated protein 1) pathway is the major regulator of cytoprotective responses to oxidative and electrophilic stress [38,39]. Nrf2 is a basic region-leucine zipper-type transcription factor that belongs to the Cap ‘n’ Collar (CNC) family of regulatory proteins that also includes NF-E2, Nrf1, Nrf3, Bach1 and Bach2 [40]. The protein contains seven functional domains, known as Neh1–Neh7. The major regulatory domain is Neh2, which is located in the N terminus of Nrf2. The Neh2 domain contains seven lysine residues critically involved in ubiquitin conjugation [41] as well as two binding sites, known as ETGE and DLG motifs, that help to regulate Nrf2 stability [42,43].

The Nrf2 protein is rapidly turned over; it has a short half-life under basal conditions (7–15 min) which is increased to 30–100 min in the presence of inducers [44,45]. The Nrf2 protein stability is primarily regulated by Keap1 [46] (Fig. 4). The ETGE and DLG motifs within Nrf2 interact with Keap1. Keap1 is a substrate adaptor protein for a Culin 3 (Cul3)-dependent E3 ubiquitin ligase complex; hence binding of Keap1 with Nrf2 mediates ubiquitination and subsequent proteasomal degradation of Nrf2 [41,46–50].

Fig. 4. Keap1 dependent regulation of Nrf2. Under basal conditions, Nrf2 is sequestered in the cytosol by a Keap1 homodimer, which facilitates the ubiquitination and proteasomal degradation of Nrf2. Inducers react with specific cysteine residues in Keap1, leading to the release of Nrf2 and allowing its nuclear translocation. In the nucleus, Nrf2 heterodimerizes with small Maf proteins and binds to the antioxidant response element (ARE), activating the expression of a battery of cytoprotective genes.
Sulfhydryl groups within Keap1 act as sensors for electrophiles and oxidants [51]. In the presence of reactive oxygen species, critical cysteine residues in Keap1 become oxidized leading to a conformational change of Keap1, which prevents its binding to Nrf2. As a consequence, Nrf2 ubiquitination and degradation is stopped, and its nuclear translocation promoted.

Among the 27 cysteine residues within Keap1, Cys151 seems to be the most critical sensor residue which mediates the formation of an intermolecular disulfide – Cys151–Cys151 – between two Keap1 molecules. Thus, in response to a diverse array of stimuli, redox-sensitive residues, especially Cys151 within Keap1, can be covalently modified, allowing Nrf2 to evade Keap1-mediated ubiquitination [52,53]. As a result, Nrf2 accumulates in the nucleus and heterodimerizes with a small Maf protein. The Nrf2-small Maf heterodimer binds to regulatory gene regions known as ARE sites with a consensus sequence 5′-TGACnnnGC-3′, also called electrophile response elements (EpRE) [54]. Substances able to induce a response at the ARE are derived from a variety of sources, including phytotoxins and derivatives such as genistein, quercetin, curcumin and sulforaphane; environmental agents like paraquat and metals and endogenous inducers such as nitric oxide, oxygen intermediates of target genes are derived from a variety of sources, in which can be phosphorylated by GSK-3β; La Autoantigen, is activated by ROS promoting dephosphorylation of Nrf2 [56,57]. ROS also regulate Nrf2 by promoting the ubiquitination of Nrf2 by disrupting Nrf2-Keap1 binding causing persistent activation of Nrf2 [56,57]. ROS also regulate Nrf2 by promoting the formation of an intermolecular disulfide bond, promoting dissociation from Keap1, and Nrf-2b activation [68]. On the other hand, inhibition of activation of NF-κB by H2O2 can be mediated by Keap1-dependent degradation of IKKβ [69]. NF-κB target genes mainly include enzymes involved in the antioxidant response such as NADP(H):quinone oxidoreductase 1 (NQO1), glutathione S-transferases (GSTs), cysteine-glutamate exchange transporter, and multidrug resistance-associated protein.

In addition to cysteine-thiol modification, several other mechanisms have been described to regulate Nrf2 signaling. For example, some proteins, such as autophagy substrate p62 and p53-regulated p21, interfere with the Keap1-dependent ubiquitination of Nrf2 by disrupting Nrf2–Keap1 binding causing persistent activation of Nrf2 [56,57]. ROS also regulate Nrf2 by promoting the Ser209 phosphorylation of eIF4E, the initiation factor 4F complex which recruits the 40S ribosome to the 5′-UTR of the Nrf2 mRNA. An additional level of regulation is provided by an Internal Ribosome Entry Site (IRES) sequence in the 5′-UTR in the Nrf2 mRNA. IRES activity is regulated by IRES trans-acting factors (ITAFs), which recruit eIFs and ribosomes to initiate translation. One of such ITAFs, La Autoantigen, is activated by ROS promoting dephosphorylation of Ser360 or phosphorylation of Thr301 by AKT. Concerning Nrf2 export from the nucleus, oxidation of Cys183 in Nrf2 inhibits its binding to the nuclear exporting protein CRM1, while the phosphorylation of Tyr568 by FYN, a kinase activated by GSK-3β strengthens this interaction [58].

Moreover, Nrf2 can bind to the E3 ubiquitin ligase β-TrCP (β-transducing repeat-containing protein) via its Neh6 domain, which can be phosphorylated by GSK-3β (glycogen synthase kinase 3β), leading to Cullin 1 (Cul1)–dependent ubiquitination and degradation of Nrf2 in a Keap1–independent manner [44,58]. GSK-3β is inhibited by phosphorylation, which can be catalyzed by AKT, ERK, p38 MAPK and PKC kinases, all known to be activated by H2O2. Also, Nrf2 cross-interacts with several other signaling pathways such as NF-κB, Akt, p53, and homodomain transcription factors, which also expands the scope of Nrf2–regulated genes [59–62]. Additionally, Nrf2 phosphorylation by protein kinase RNA (PKR)-like ER kinase (PERK) or PKC-δ, both activated by ROS, causes dissociation of the Nrf2/Keap1 complex.

Post-translational modifications at the level of Keap1 that prevent its interaction with Nrf2 are another mechanism leading to Nrf2 activation. Indeed, Keap1 phosphorylation at Tyr141 renders the protein highly stable and its dephosphorylation induced by hydrogen peroxide results in rapid Keap1 degradation and Nrf2 activation [63]. Interestingly, Keap1 knockout mice die short after birth, but the lethal phenotype can be reversed by the double knockout of Keap1 and Nrf2 [64], which suggests that either excessive or insufficient Nrf2 activity may be detrimental for normal physiology. In fact, the constitutive activation of the Nrf2 pathway can be beneficial for tumor survival [65].

3.2. NF-κB

The NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells)/REL family of transcription factors consist of homodimers of five distinct proteins, the REL subfamily proteins (p65/RELA, RELB, and c-REL) and the NF-κB subfamily proteins (p50, and p52, and its precursors p105 and p100, respectively). All NF-κB/REL proteins contain a Ref-1/homology domain (RHD), which is responsible for dimerization, recognition and binding to DNA as well as interaction with the inhibitory κB (IκB) proteins. The IκB family is composed of IκB-α, IκB-β, IκB-ε, IκB-γ, and BCL-3, all of which possess typical ankyrin repeats that bind to the RHD of NF-κB/REL proteins to interfere with their function. The IκB–kinase complex (IKK complex) catalyzes phosphorylation of Iκ Bs with the result that Iκ Bs are targeted for degradation by the 26S proteasome thereby freeing NF-κB. The latter then binds to the DNA consensus sequence 5′-GGGRNNYYCC-3′ (R is a purine, Y is a pyrimidine, and N is any base) in the promoter/enhancer regions of target genes [66].

Activation of IKK occurs also by phosphorylation and is catalyzed by an IKK kinase, including TGF-β-activated kinase (TAK1), AKT, or NF-κB inducing kinase-1 (NIK1), all of which can be regulated by H2O2 [66]. Alternatively, the reduced form of the dynamin light chain protein LC3 binds to IκB and inhibits its phosphorylation by IKKs. H2O2 induces dimerization of LC3 by a disulfide bond, promoting dissociation from IκB, and NF-κB activation [68]. On the other hand, inhibition of activation of NF-κB by H2O2 can be mediated by Keap1-dependent degradation of IKKβ [69]. NF-κB target genes mainly include enzymes involved in the antioxidant response such as ferritin heavy chain [70] and SOD2 [71]. Another NF-κB target gene that contributes to both survival and innate immune functions is the HIF-1α gene, encoding the oxygen-regulated subunit of the hypoxia responsive transcription factor HIF-1 [72]. On the other hand, hydroquinone and tert-butyl hydroquinone, prototypes of phenolic antioxidants, block lipopolysaccharide (LPS)-induced transcription of TNFα and NF-κB as they block the formation of NF-κB/DNA binding complexes [73].

3.3. AP-1

AP-1 (activator protein 1) is a family of dimeric bZIP transcription factors including Jun, Fos, ATF/CREB, JDP and MAF that usually form heterodimers that bind to a TPA-responsive element (TRE, 5′-TGAG/CTCA-3′) or cAMP response elements (CRE, 5′-TGACGCTCA-3′). They regulate several cellular processes, including cell proliferation, apoptosis, survival, and differentiation. AP-1, when upregulated, concentrates in the nucleus to activate gene expression [74]. H2O2 was shown to induce transcription of both c-JUN and c-FOS via activating the JNK, p38 MAPK and ERK signaling cascades [75], while antioxidants like butylated hydroxyanisole (BHA) and pyrrolidine dithiocarbamate (PDTC) induce AP-1 binding activity and AP-1-dependent gene expression including glutathione S-transferase [76].

Although several other pathways can be regulated by ROS (see above), the primary responses against different intensities of intermediate oxidative stress are mainly modulated by the cooperation of the three pathways: NF-κB, AP1 and MAP kinases. However, with increasing oxidative stress, the Nrf2/ Keap pathway (see above) is also needed to induce antioxidant defenses and to minimize oxidative damage. When these defenses are not strong enough to contend against oxidative stress, the cell induces...
formation/opening of the mitochondrial permeability transition pore, as an efficient way to decrease ROS production, by decreasing the mitochondrial membrane mitochondrial potential. However, this increases the oxidative state in the cell [77,78], resulting in a homeostatic disruption of the redox balance, cell damage, and apoptosis [79].

3.4. Role of microRNAs in antioxidant responses

MicroRNAs are endogenous non-coding RNAs. They are small single-stranded RNAs (22–26 nt) and are now considered essential post-transcriptional regulators of gene expression. They bind to the 3′-UTR region of mRNAs by Watson-Crick complementarity and through different molecular interactions, they are able to block their translation into proteins. In 1998, Craig Mello and Andrew Fire showed how the formation of double stranded RNA in C. elegans produced transcriptional silencing and they described the first microRNA named Lin-4 in this same model [80,81]. During the last decades knowledge in this field has grown exponentially in such a way that a recent update of the main miRNA database (miRbase 21, June 2014) [82], listed 28,645 miRNAs in more than 142 species. The vast majority of these microRNA are highly conserved across species, including non-vertebrate ones as Drosophila or C. elegans.

MicroRNA transcription depends on its localization across the genome. These small RNAs can be encoded by intronic regions (about 80%) [83], intergenic regions, non-coding genes or even exons [84]. Transcriptional regulation of microRNAs is a complex process and has been reviewed in depth elsewhere [85,86]. miRNAs are also processed sequentially in the nucleus and cytoplasm by specific proteins finally leading to the generation of a mature miRNA (for review see [85,87]).

In the last years miRNAs have been found to regulate stress responses including redox balance, to the extent that the term “redoximiRs” has been coined to define a subset of miRNAs that either regulate redox pathways or are themselves regulated by the redox status [88]. As an example, a significant number of publications describe miRNAs that might target the Nrf2 pathway or that of its co-regulators Keap1 and Bach1 (Table 1) [88–103]. Also, we have recently described one miRNA, miR-433, which targets GSH biosynthetic enzymes in an Nrf2-independent manner, resulting in decreased GSH levels and antioxidant capacity with important implications for organ fibrosis [104]. Of interest Yang et al. have described Nrf2 and GSH regulation in hepatic fibrosis produced by miR-27b, a microRNA modulated by c-Myc [96].

In the case of Dicer, one of the endonucleases involved in miRNA maturation, several publications have reported its down-regulation in aging, senescence and stress models, hence altering the abundance of mature miRNA number [105,106]. Dicer down-regulation can be prevented by the use of exogenous molecules with antioxidant potential, such as resveratrol and sulforaphane, both known to enhance Nrf2 activation. This is consistent with the fact that Dicer bears ARE sequences in its promoter region, which are conserved in human and murine species [105]. The NF-κB family of transcription factors (see above) is also able to regulate acute stress responses during inflammation through their interaction with miRNAs, specifically by upregulating the expression of miR-9, miR-155 and miR-146. Interestingly, some of the validated target genes for these three miRNAs are also pro-inflammatory molecules themselves [107]. Thus, these miRNAs may contribute to limit the duration of acute stress responses and reduce their related damage [108].

MicroRNAs are also susceptible of suffering epigenetic modifications that modulate their expression. Reciprocally, epigenetic enzymes like histone deacetylases (HDAC) and DNA-methyl transferases are also targets of oxidative stress and hence changes in their activities may result in changes in miRNA expression [109,110].

The study of miRNAs in pathological contexts has been useful to link them with new targets including enzymes professionally dedicated to ROS generation such as NOXs. For example, NOX4 has been reported to be regulated by miR-25, miR-146a and miR-23a [111]. Moreover other miRNAs, as miR-210 and miR-128a, have been identified in mitochondria as capable of regulating their metabolic activity [112]. These mitomiRs may play a role in the initiation and progression of cardiovascular disease (Table 1) [113–118]. Regarding other stress conditions, the unfolded protein response or endoplasmic reticulum (ER) stress (see below) includes an adaptive response whereby some miRNAs are modulated or regulate ER signaling. For example ER stress induces changes in the localization of AGO proteins, one of the essential set of proteins in the RISC complex, suggesting a miRNA repression role during ER stress [119]. However, other miRNAs are upregulated in these conditions [120]. In addition, other environmental stresses such as hypoxia also induce a specific subset of miRNAs (hypoximiRs), some of them involved in antioxidant responses [121–125].

Among these, miR-155, miR-210, miR-200 or miR-24 are related to the archetypal hypoxia-regulated transcription factor Hif-1α. In the context of ischemia–reperfusion (see below) miR-1 and miR-133 have been described in the heart in the setting of arrythmia [124,126] while the miR-34a/Sirt1 pathway has been reported to be protective [127] (Table 1).

### Table 1

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4. Antioxidant responses in specific pathophysiological contexts

Obviously, antioxidant responses are involved in an enormous amount of cellular and tissular processes in relation to both physiology and pathophysiology. Excellent reviews covering these issues are already available [8,128–130]. For the sake of clarity we now discuss in depth two examples, one related to a cellular
biochemical process, ER stress and the other one in the context of a relevant pathophysiological common event, ischemia–reperfusion.

4.1. ER stress

The endoplasmic reticulum (ER) has two principal major functions. First, it is the primary site of protein synthesis and packaging, second, it plays a central role in metabolism and various signaling processes. The synthesis and the packing of proteins within the ER is strongly coupled to native disulfide bond formation. Correct disulfide bond formation is coupled to oxidation, reduction and isomerisation of disulfide bonds within the respective proteins. Indeed, there is growing evidence that a large number of different gene families and redox carriers play a role in the supply of redox equivalents to achieve a correct protein disulfide bond formation.

The rate-limiting step for disulfide bond formation is late-stage isomerization, which is necessary to achieve a substantial regular secondary protein structure. Since protein folding is not error-free, correction of incorrect disulfide bonds requires a partial unfolding of the already formed structures. Remarkably, proper folding and disulfide bond formation of proteins appears to be dependent on the redox status within the ER. Although reducing conditions prevail in the cytosol, the lumen of the ER represents a more oxidizing environment, with a higher ratio of oxidized to reduced glutathione [GSSG/GSH] [131]. Especially, reduced glutathione (GSH) or protein thiols react with ROS and thus contribute to cellular redox homeostasis. In particular, GSH is considered as the major thiol-disulfide redox buffer of cells and often the ratio between GSSG and GSH is used to determine the redox state. In the cytoplasm, the ratio of reduced glutathione to oxidized glutathione is \(\approx 50:1\) [132]. By contrast, this ratio is 1:1 to 3:1 in the ER. It appears that the oxidizing conditions are mainly important for de-novo disulfide bond formation of most proteins synthesized in the ER. However, disulfides can also be reduced in the ER, and hence redox homeostasis must be maintained.

In general the factors catalyzing disulfide bond formation become reduced and in order to fulfill further catalytic cycles these factors need to be re-oxidized [133]. In eukaryotic cells, in particular mammalian cells, it is believed that molecular oxygen functions as the final electron acceptor. Within that system, the ER resident oxidoreductase Ero1 and protein disulfide isomerase (PDI) appear to play key roles [134,135]. Overall, this implies that disturbances in the nutrient and metabolic flux are important effectors of Ero1 and PDI function and could thereby contribute to the generation of ROS and the build-up of an ER stress response.

The ER stress or unfolded protein response (UPR) is in general triggered when the ER function is diminished, e.g. when the entry of newly synthesized proteins exceeds the folding capacity. The accumulation of unfolded proteins within the ER leads to an activation of signaling events with the aim to increase the folding capacity and to reduce folding load. The folding capacity is enhanced after ER enlargement [136] and synthesis of ER resident molecular chaperones and foldases [137]. Decreasing expression of genes encoding secretory proteins [138] as well as increasing the ER-associated degradation (ERAD) of slowly folding or misfolded proteins [139] reduces the folding load. Three distinct branches of the ER stress response are in principle distinguished: the double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) branch, the inositol requiring kinase 1 (IRE1) branch, and the activating transcription factor 6 (ATF6) branch. All three components associate with the ER luminal chaperon BiP (also known as glucose-regulated protein 78, GRP78; or Kar2p in yeast). Once unfolded proteins accumulate in the ER, BiP preferentially associates with the unfolded proteins instead of PERK, ATF6, and IRE1, resulting in activation of their downstream signalling molecules [133]. So far, the cross-talk between ROS and ER stress appears to be most effective for the PERK branch. Activation of PERK promotes phosphorylation of the α-subunit of eukaryotic translation initiation factor 2 (eIF2α) [140] with the result of a block in protein translation-initiation except that of ATF4. ATF4 in turn induces expression of genes like growth arrest and DNA damage-34 (GADD34), and CAAT/Enhancer binding protein (C/EBP) homologous protein (CHOP) [141]. In addition, another direct PERK target appears to be Nrf2 (see above). Interestingly, Nrf2-/− cells are very sensitive to ER stress [142] implying that ER stress via PERK phosphorylates Nrf2, which, after heterodimerically partnering with other transcription factors, activates transcription through ARE binding as detailed above.

Although a redox unbalance cannot be caused by affecting the PERK branch, most ROS in the ER appear to be generated via Ero1 [143,144]. In humans, there are two ERO1 isoforms, hERO1-La and hERO1-Lb [145–147], which contain flavin adenine dinucleotide (FAD). Indeed, overexpression of both Ero1 proteins shifts the redox state of PDI towards the oxidized form (Fig. 5), which thus also influences oxidation of PDI substrates [148,149]. The isoform Ero1La was also localized in the mitochondria-associated membrane (MAM), and this localization depends on the oxidizing conditions within the ER [150]. During disulfide bond formation electrons pass several thiol-disulfide exchange reactions, the thiols of the substrate, PDI and Ero1 from where they finally reach molecular oxygen [143]. In a simplistic view, Ero1 usually donates a disulfide bond to PDI and at the same time the receiving electrons are passed on via Ero1’s FAD moiety to reduce oxygen and to generate hydrogen peroxide (Fig. 5) [151,152]. Interestingly, the hydrogen peroxide produced can be used by the ER-localized peroxiredoxin 4 to oxidize PDI and some PDI family members. This process does not only increase the efficiency of the Ero1-driven disulfide bond formation but also allows disulfide bond formation via alternative sources of \(\text{H}_2\text{O}_2\) [153,154]. Although these findings underline the importance of hydrogen peroxide for proper disulfide bond formation, the incomplete reduction of oxygen during this process results in the formation of superoxide anion radicals, which can then be dismutated to \(\text{H}_2\text{O}_2\) or converted to other ROS.

In addition, removal of superoxide by SODs appears to be important for ER function. This is supported by findings from mice transgenic for a non-functional Cu/Zn-superoxide dismutase mutant. These mice show protein aggregate formation and extensive dilatation of the ER along with degeneration of motor neurons of the spinal cord, features also seen in patients with amyotrophic lateral sclerosis [155–157]. Whether the ER dilatation observed in these
mice is the direct effect of ROS on protein folding or the more indirect result via activation of signalling events is not known yet. However, the Cu/Zn-superoxide dismutase-induced degeneration of neurons could be blocked by overexpression of the cytosolic chaperone heat-shock-protein 70 (HSP70) [158,159] suggesting that cytosolic ROS may affect protein folding in the ER. This is further supported by the finding that mutant CuZn-superoxide dismutase induces BiP (GRP78) [156].

The connection between oxygen, ROS, energy metabolism and ER resident proteins is also underlined by the fact that some of the latter, known as glucose-regulated proteins (GRPs) [160], are induced by hypoxia [155,161,162]. In line with this, severe hypoxia or anoxia can also activate ATF4 [163,164]. Although ER stress and anoxia induce ATF4, the latter was shown to be independent of hypoxia-inducible transcription factors (HIFs). However, the link between ER function and regulation of the O2-sensitive HIF-1α subunit seems to be independent of ATF4 since HIF-1α localizes to the perinuclear ER membrane under normoxia, where ROS such as hydroxyl radicals are generated in an iron-dependent Fenton reaction [165]. Moreover, the so called transmembrane proline 4 hydroxylase (P4HTM), one member of the four HIF-proline hydroxylases, which are critically involved in the regulation of HIF protein stability was found to be an ER resident enzyme [166].

Interestingly, from the two mammalian Ero members only Ero1Lα but not Ero1Lβ appears to be under transcriptional control from HIF-1 [167]. Thus, ROS-dependent HIF-1α regulation may be part of a feedback cycle contributing to thiol-oxidation in the ER via adaptation of Ero1Lα transcription to the overall cellular redox status.

However, ER-derived ROS, e.g. due to the action of Ero1, may not only have an intricate role in disulfide bond formation but also for the regulation of cell death and life span. This is in line with data showing that ER stress induces accumulation of ROS in PERK or ATF-4 knockout cells to higher levels than in wild-type cells [144] and that this contributes to apoptosis and a reduced life span in C. elegans. Interestingly, repression of Ero-1 restored normal life span in C. elegans with repressed PERK expression [144]. Furthermore, the cytokine TNFα caused cell death in a ROS- and ER stress dependent fashion in murine fibrosarcoma L929 cells [168]. However, pre-conditioning these cells with the ER stressor tunicamycin substantially inhibited TNFα-induced ROS production and cell death. Within that scenario, the tumor necrosis factor receptor-associated factor 2 (TRAF2) was found to interact with the cytosolic domain of IRE1α [170,171]. This trimeric complex can be activated by ROS involving thioredoxin, another interacting part- ner of ASK1 [172–174]. Oxidative stress disrupts the ASK1-thioredoxin complex by oxidation of thioredoxin and thereby activating ASK1 [173] and subsequently JNK, p38 and cell death [175]. Interestingly, the Jun activation domain-binding protein (JAB-1, also known as COP9 signalosome subunit 5) may be a negative feed- back regulator since it was shown that it interacts with IRE1α [176]. Thus, ER stress and oxidative stress may induce cell death by using the same molecular complex consisting of IRE1α/TRAF2/ASK1/thioredoxin with JAB1 acting as negative regulator.

4.2. Ischemia-reperfusion

The process of ischemia followed by reperfusion is often accompanied by the activation of an injurious cascade. Although the
pathogenesis of ischemia–reperfusion (IR) is not completely understood, there is considerable evidence implicating ROS as an initial cause of the injury. Ischemia results in a build-up of NADH that, upon reperfusion, generates a burst of free radicals from the electron transport chain (ETC). This can adversely alter proteins and DNA, and further propagate lipid radical reactions that produce electrophile species.

4.2.1. Post-translational modifications of mitochondrial proteins in ischemia–reperfusion

The specific modifications of mitochondrial proteins leading to improved mitochondrial function have the potential to modulate cardiac IR damage. The metabolism of (NO) can lead to the S-nitrosylation of proteins containing thiol groups, including mitochondrial proteins, thereby reversibly regulating their activity. In particular, complex I, the entry point for electrons from NADH into the respiratory chain, can be S-nitrosylated [177–179], which may alter its activity and thereby protect against damage during IR injury [180,181]. In agreement with this, mitochondria-targeted S-nitrosothiols (MitoSNO), which induce functional modification of mitochondrial proteins following S-nitrosylation, protect against heart IR injury [182–184]. This modification of complex I appears to increase superoxide production from the complex [178,185]. However, in the context of IR, reversible S-nitrosylation of complex I, in particular, a specific Cys residue (Cys 39) within ND3 (Fig. 6), slows the reactivation of mitochondria during the first minutes of reperfusion, thereby decreasing ROS production, oxidative damage and tissue necrosis [184]. In addition, consistent with their potential role in cardioprotection, S-nitrosothiols were detected in mitochondria isolated from perfused rat hearts subjected to ischemic preconditioning [177,186].

Besides S-nitrosylation, deacetylation of mitochondrial proteins has been reported to attenuate IR damage. The sirtuin family are NAD+-dependent protein deacetylases that mediate the effects of caloric restriction [187]. It has been shown that caloric restriction primes cardiac mitochondria for ischemic stress by deacetylating specific mitochondrial proteins of the ETC [188], suggesting that deacetylation of specific mitochondrial proteins by sirtuin preserves mitochondrial function and attenuates myocardial damage during IR.

4.2.2. The Nrf2 pathway in ischemia–reperfusion

Since the generation of reactive species has been involved in the pathogenesis of IR, reducing oxidative stress is a potential therapeutic approach to prevent IR injury. Nrf2 accumulates in the nucleus after IR in cardiac [189] and renal tissues [190]. Moreover, several compounds that activate the Nrf2 pathway are protective against IR damage. Thus, curcumin, a natural compound in turmeric with antioxidant activity via the Nrf2 pathway, protects neurons from death caused by oxygen and glucose deprivation in an in vitro model of IR [191], and exerts beneficial effects against cardiac [192] and renal [193] IR injury. Likewise, mono-carbonyl analogues of curcumin such as 14p have been reported to decrease oxidative stress and limit myocardial IR injury via Nrf2 activation [194]. In addition, sulforaphane, a compound obtained from cruciferous vegetables, which induces Nrf2-dependent gene expression, attenuates liver injury induced by intestinal IR in rats [195], protects neurons and astrocytes from delayed cell death induced by oxygen and glucose deprivation [196,197], reduces the infarct size of isolated perfused rat hearts subjected to IR [198], and prevents renal injury caused by IR in vivo and in vitro [199]. This compound also protects rat hearts from early injury after experimental transplantation [200]. Taken together, these observations indicate that the activation of the Nrf2 pathway protects against IR injury and is a potential target to ameliorate this type of damage.

4.2.3 Lipid peroxidation products in cardioprotection against ischemia–reperfusion

As a result of oxidative damage, lipid peroxidation products accumulate in the heart. Mitochondria are both a primary source and target of lipid peroxidation products [201]. Reactive aldehydes such as 4-hydroxy-2-nonenal (4-HNE) are formed during cardiac IR. High concentrations of aldehydes are responsible for much of the damage produced during IR. In fact, aldehyde dehydrogenase 2 (ALDH2) has been reported to protect myocardium against IR injury through detoxification of aldehydes [202]. By contrast, low concentrations of aldehydes can stimulate stress resistance pathways to confer cardioprotection. Thus, lipid peroxidation products are involved in the transcriptional regulation of endogenous antioxidant systems. Recent evidence demonstrates that transient increases in lipid peroxidation products may be beneficial in cardioprotection by contributing to mitohormesis (i.e. induction of antioxidant systems) in cardiomyocytes. Thus, 4-HNE activates Nrf2 in the heart and increases the intramyocardial GSH content, improving the functional recovery of the left ventricle following IR in isolated perfused hearts [203]. 4-HNE also induces mitochondrial uncoupling protein 3 (UCP3) via Nrf2 in cardiomyocytes, promoting cell survival [204].

5. Signal transduction in the context of antioxidant responses: some key players

Historically considered as cellular damaging agents, ROS have emerged as important modulators of intracellular transduction signaling. ROS interact with redox-sensitive signaling molecules including transcription factors, protein tyrosine phosphatases, protein kinases and ion channels, that contain cysteine residues whose SH groups are oxidized, causing a change in their biological activity, regulating cellular processes like growth factor signaling, hypoxic signal transduction, autophagy, immune responses, and stem cell proliferation and differentiation [129,205].

These redox changes include activation/deactivation cycles of redox metabolism and expression of antioxidant enzymes expression that are essential to maintain nucleoflic tone and oxidant/antioxidant balance in the cells [130]. In the following section a brief overview of some examples of redox-sensitive cytosolic and mitochondrial proteins is given.

5.1. Cytosolic regulation

5.1.1. Protein tyrosine phosphatases

One of the most studied direct targets of ROS signaling is the family of protein tyrosine phosphatases. All tyrosine phosphatases have a conserved 230-amino acid domain that contains a reactive and redox-regulated cysteine, which catalyzes the hydrolysis of protein phosphotyrosine residues by the formation of a cysteiny1-phosphate intermediate, that later is hydrolyzed by an activated water molecule. Because of the unique environment of the tyrosine phosphatase active site, the catalytic cysteine presents an unusually low pKa value and is therefore deprotonated at physiological pH, existing as a thiolate anion (Cys−S−). Oxidation of this residue to sulfenic acid by H2O2 renders the tyrosine phosphatases inactive. This oxidation of cysteine to sulfenic acid is reversible, while oxidation by the addition of two (sulfenic acid) or three (sulfonic acid) oxygens to the active site cysteine is irreversible. These modifications can be further stabilized by the formation of inter or intramolecular disulfide (S–S) or sulfenyl–amide bonds. Thus ROS significantly inhibit activity of tyrosine phosphatases, resulting in increased tyrosine phosphorylation [206]. For instance, redox regulation of protein tyrosine phosphatase 1B involves a sulfenyl–amide intermediate, also accompanied by large
conformational changes in the catalytic site that inhibit substrate binding. This is an unusual protein modification that protects the active-site cysteine residue of PTP1B from irreversible oxidation [207,208]. Interestingly, S-glutathionylation of the sulfenic acid form of these proteins is proposed as a mechanism to protect against irreversible oxidation [209].

5.1.2. Protein Kinases

Protein kinases are a group of enzymes that phosphorylate tyrosine, threonine, and/or serine residues of target proteins altering their function. Susceptible cysteines in some serine/threonine protein kinases are directly modified by ROS. Protein kinase C (PKC) contains a cysteine rich domain susceptible to oxidation, while oxidation of Cys-245 and Cys-487 in the kinase domain of the nonreceptor tyrosine kinase Src results in the activation of the protein [210]. Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases that play a central role in coupling various extracellular signals to a variety of biological processes, such as gene expression, cell proliferation, differentiation, and cell death. The activity of MAP kinases (ERK, c-Jun and p38) is regulated by phosphorylation cascades: MAPKs activation is induced through the phosphorylation of their threonyl and tyrosyl residues within a tripeptide motif TXY by a dual specificity kinase termed MAP kinase kinase (MKK), which in turn is phosphorylated and activated by an upstream kinase called MAPK kinase kinase (MAP3K) [211]. However, MAPKs can also be activated by ROS.

In vascular smooth muscle cells, intracellular ROS are critical for AII-induced activation of p38MAPK, JNK and ERK5, whereas phosphorylation of ERK1/2 activation in smooth muscle cells is redox sensitive, in contrast to fibroblasts, suggesting that redox-regulation of MAP kinases may be ligand- and cell-specific [212].

Many growth factor and cytokine receptors bearing cysteine-rich motifs can be targets of oxidative stress. Thus, insulin-like growth factor-I (IGF-I) activates ERK pathway through ROS-mediated activation of EGF receptor, which by mediation of Ras-GTPase activates Raf and MEK, phosphorylating ERK [213]. ROS may also activate MAPK pathways through the oxidative modification of the intracellular kinases. ASK-1, a member of the MAP3K superfamily for JNK and p38, binds to reduced thioredoxin in nonstressed cells. Upon oxidative stress, thioredoxin becomes oxidized and dissociates from ASK-1, leading to activation of JNK and p38 pathways through oligomerization of ASK-1 [214].

Another mechanism for MAPK activation by ROS includes the inactivation and degradation of the MAPK phosphatases (MKPs). Kamata et al. reported that H2O2 inactivates MKPs by oxidation of their catalytic cysteine, which leads to sustained activation of the MAPK pathway [71]. However, Zhou et al. found that upregulation of MKP-1 expression by H2O2 correlates with inactivation of JNK and p38 activity [215]. This paradox in the roles of ROS as “inducers” in the regulation of MKP-1 expression and as “inhibitors” may be, at least in part, related to differences in the concentrations of ROS and of the redox sensitivity of Cys residues within MAPK phosphatases.

Some antioxidant responses are also regulated by MAPKs signaling. Activation of mitogen activated protein kinase pathways induces antioxidant response element-mediated gene expression via a Nrf2-dependent mechanism [214]. Prx I is an antioxidant that is up-regulated in a ROS/p38 MAPK-dependent manner [217]. In addition, MAPK regulates NK-κB activation by modulating the phosphorylation of IkB-alpha and p65 NF-κB, stimulating iNOS and AP1 gene expression (Fig. 7) [218]. It also has been reported that mitogen-activated protein kinases (MAPKs) are involved in some polyphenol-mediated regulation Phase II enzymes gene expression and induction of apoptosis [219].

**Fig. 7.** Induction of antioxidant responses by ROS-mediated activation of cytosolic cell signaling pathways. ROS drive activation of MAPKs. ERK and JNK are involved in recruiting c-Fos and c-Jun to the nucleus where they activate the transcription factor AP-1, whereas activation of p38 and IKK is important in the transcriptional activation of NF-κB. Both of these factors are important in regulating diverse genes, including antioxidants (p53, NQO1, GSTs, SOD2, Ap-1,p53, and Prx I).
5.2. Mitochondrial regulation

Mitochondria are regulated in an intricate manner by oxidants and electrophiles. The activity of several complexes of the electron transport chain is modulated by post-translational modifications such as S-nitrosylation, S-glutathionylation or electrophile additions [222]. As mentioned above, complex I (NADH ubiquinone oxidoreductase) is modified by nitric oxide or its derivatives [177,178,185,220,221] and glutathione [222–225]. The S-nitrosylation of complex I correlates with a significant loss of activity that is reversed by thiol reductants. S-nitrosylation was also associated with increased superoxide production from complex I [178,185]. The fact that mitochondrial superoxide formation can be regulated by S-nitrosylation of complex I may play an important role in mitochondrial redox signalling [226]. Complex I has two transitional states, the active (A) and the inactive (D) states, and the complex is S-nitrosylated in the D state [179]. The A to D transition may take place during hypoxia and this might be important in the setting of ischemia–reperfusion. Other studies have reported nitrotyrosine modification on the complex [227,228]. In addition, reversible glutathionylation of complex I increases mitochondrial superoxide production [222]. Other electron transport complexes, in particular, complex II (succinate dehydrogenase) and complex V (ATP synthase), have been shown to be modiﬁed by reactive species [229,230]. Likewise, mitochondrial matrix proteins such as NADP⁺–isocitrate dehydrogenase [231,232], α-ketoglutarate dehydrogenase [233,234] and aconitase [230,235], as well as proteins of the intermembrane space such as creatine kinase [236] and cytochrome c [237] can also be modiﬁed by reactive species, affecting in most cases their catalytic activity.

In response to stress or to an increased demand in energy, mitochondria produce a limited amount of ROS that act as signalling molecules, initiating a molecular stress response that leads to transcriptional changes in the nucleus. A transient ROS signal generates an endogenous response that allows detoxiﬁcation of ROS by inducing defence enzymes such as superoxide dismutase or catalase, as well as other stress defence pathways [238]. This process is called retrograde response [239,240]. The release of ROS from mitochondria can function in numerous signalling pathways [241]. Some examples are the regulation of cytosolic stress kinases [71], modulation of hypoxic signalling [242], and activation of macroautophagy [243].

In contrast to this function of ROS as signalling molecules, sustained high levels of ROS can cause intracellular damage. This nonlinear response to mitochondrial ROS is known as mitochondrial hormesis or mitohormesis [240]. This concept considers ROS as essential signalling molecules, and not just harmful and damaging by-products of mitochondrial metabolism.

5.2.1. Targeting mitochondria to eliminate ROS production

Antioxidants can be targeted selectively to mitochondria to reduce oxidative stress. Mitochondria-targeted antioxidants comprise a triphenylphosphonium cation (TPP⁺) covalently attached to an antioxidant, which enables mitochondrial delivery of the compound. The most extensively studied is MitoQ, which contains the antioxidant ubiquinol covalently attached to TPP⁺. MitoQ decreases cardiac [244] and renal [245] IR injury and it is therefore a potential therapeutic agent against this type of damage. Ongoing clinical trial are evaluating the efﬁcacy of Bendavia, Szeto–Schiller peptides that also improve post-infarction cardiac function and post-IR kidney injury post-IR [246–248]. Another strategy to eliminate harmful ROS generation is the removal of damaged mitochondria, which can produce excessive ROS. Recent studies have focused on the inhibition of Drp1 (dynamin-related protein 1), a large GTPase involved in mitochondrial fission. In particular, the inhibitor mdivi-1 reduces myocardial infarct size in mice undergoing IR, and reduces excessive fragmentation in adult rat cardiomyocytes exposed to simulated IR injury [249]. In addition, a small separation of function peptide, P110, which speciﬁcally affects the interaction between Drp1 and fission 1, diminishes excessive mitochondrial fission and reduces neurotoxicity in cells derived from patients with Parkinson disease [250]. Another mechanism to prevent pathological ROS production consists of eliminating damaged mitochondria. After cellular insults such as oxidative stress caused by IR, the elimination of damaged or dysfunctional mitochondria through autophagy (mitophagy) is upregulated by diverse molecular mechanisms [251,252], contributing to the maintenance of mitochondrial quality control. Finally, other mechanisms that maintain mitochondrial quality control are the unfolded protein response and the proteasome machinery, which are also potential therapeutic targets.

6. Conclusion

Since the discovery of ROS there has been increased interest and research into the processes of ROS generation, ROS-mediated effects and functional consequences of ROS driven processes. We now have a relatively comprehensive view of how ROS can be generated, converted into each other and how their action affects lipids, proteins and DNA. A concept that has gained momentum in redox biology and is probably to remain for a long time is the major importance of hydrogen peroxide as the paradigmatic signalling molecule [253]. However, we still need more knowledge about how this signalling proceeds, the antioxidant responses involved and their thresholds to redox perturbations. Moreover, mechanisms of how ROS may be transported or interconverted inside or outside cellular compartments and how these conversions contribute to cellular homeostasis are lacking. The next few years may well provide further examples or may uncover new or alternate mechanisms contributing to redox balance, the antioxidant defense and ROS/peroxide signaling.

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