Redox compartmentalization in eukaryotic cells

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Abstract

Diverse functions of eukaryotic cells are optimized by organization of compatible chemistries into distinct compartments defined by the structures of lipid-containing membranes, multiprotein complexes and oligomeric structures of saccharides and nucleic acids. This structural and chemical organization is coordinated, in part, through cysteine residues of proteins which undergo reversible oxidation-reduction and serve as chemical/structural transducing elements. The central thiol/disulfide redox couples, thioredoxin-1, thioredoxin-2, GSH/GSSG and cysteine/cystine (Cys/CySS), are not in equilibrium with each other and are maintained at distinct, non-equilibrium potentials in mitochondria, nuclei, the secretory pathway and the extracellular space. Mitochondria contain the most reducing compartment, have the highest rates of electron transfer and are highly sensitive to oxidation. Nuclei also have more reduced redox potentials but are relatively resistant to oxidation. The secretory pathway contains oxidative systems which introduce disulfides into proteins for export. The cytoplasm contains few metabolic oxidases and this maintains an environment for redox signaling dependent upon NADPH oxidases and NO synthases. Extracellular compartments are maintained at stable oxidizing potentials. Controlled changes in cytoplasmic GSH/GSSG redox potential are associated with functional state, varying with proliferation, differentiation and apoptosis. Variation in extracellular Cys/CySS redox potential is also associated with proliferation, cell adhesion and apoptosis. Thus, cellular redox biology is inseparable from redox compartmentalization. Further elucidation of the redox control networks within compartments will improve the mechanistic understanding of cell functions and their disruption in disease.

1. INTRODUCTION

The compartmentalization of peroxide-metabolizing systems within peroxisomes has long been recognized to provide a means to utilize hydrogen peroxide as a metabolic reagent while at the same time protect redox-sensitive cell components in other cell compartments from oxidative damage. Accumulating knowledge of redox characteristics in mitochondria, cytoplasm, nuclei and the secretory pathway indicate that unique redox characteristics have evolved with each of the major compartments in mammalian cells.

Elucidation of details has been advanced by improved sensitivity and spatial resolution of redox indicators in living cells but remains somewhat limited by difficulties in calibration and ability to confirm with fractionation due to auto-oxidation and redistribution artifacts. However, methods are progressively being improved so that an understanding of subcellular redox characteristics is emerging [1,2]. In particular, development of redox western blot techniques...
for measuring redox states of proteins [3,4] has allowed quantification of steady-state redox potentials of proteins in different subcellular compartments [2,4,5]. By creating constructs to target accumulation of proteins in specific compartments, systematic mapping of redox potentials in different compartments has become possible. Targeting has been achieved using mitochondrial localization sequences (MLS), nuclear localization signals (NLS), nuclear export signals (NES), and endoplasmic reticulum retention signals. The approaches have been complemented by techniques to measure redox potential in living cells, largely based upon partitioning of lipophilic cationic fluorescent chemicals into mitochondria dependent upon the high mitochondrial membrane potential ($\Delta\psi$) [6] and development of redox-sensitive forms of green fluorescent protein [7]. Detection of disulfide reduction in the endosome has also been achieved by use of FRET [8]. Application of these methods to diverse systems has begun to clarify structural and chemical aspects of cellular functions which depend upon thiol/disulfide redox elements.

2. Mitochondrial redox regulation

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. The predominant electron transfer occurs through a central redox circuit which uses the potential energy available from oxidation of various metabolic substrates (e.g., pyruvate, fatty acids) to generate ATP. The mitochondrial inner membrane contains 5 multiprotein complexes with central functions in oxidative phosphorylation. Three of these complexes are redox-driven proton pumps: complex I (NADH-quinone oxidoreductase), complex III (coenzyme Q: cytochrome reductase), and complex IV (cytochrome oxidase). Complex II (succinate dehydrogenase) transfers electrons into the chain from succinate, and complex V (ATP synthase), is a reversible proton pump which uses the electrochemical proton gradient ($\Delta \mu_H^+$) to drive ATP synthesis.

Regulation of this process is central to cell function because cells must produce ATP while at the same time maintaining an appropriate homeostasis in terms of supply of non-essential amino acids, eliminating excess amino acids, supplying glucose and interconverting energy precursors to allow for long-term energy supply in the face of variable and intermittent food intake. Part of the regulation appears to occur through a continuous low rate of reactive oxygen species (ROS) generation, which regulates the magnitude of $\Delta \mu_H^+$ through control of Fe-S containing dehydrogenases and uncoupling proteins [9]. The molecular sensors and associated redox circuitry for this regulation remain poorly defined, but such regulation requires a specialized redox environment. In addition, the molecular machinery required for oxidative phosphorylation is highly dependent upon critical cysteine residues of proteins for enzymatic and transport activities [10], and the mitochondrial genome is susceptible to oxidative damage [11,12]. These processes are directly relevant to mitochondrial oxidative stress-related diseases such as Parkinson’s disease [13], Friedreich’s ataxia [14], Huntington disease [15], and [16], and improved understanding can be expected to aid development of improved therapies for these diseases.

2.1 Oxidants in mitochondria

ROS, principally superoxide anion radical ($O_2^-$) and its dismutation product $H_2O_2$, are derived from several sources in mitochondria. Within the electron transport chain, Complex I and III are the main sites of electron transfer to $O_2$ to produce $O_2^-$ [17]. Inhibitors of complex I and III, rotenone and antimycin A, respectively, stimulate $O_2^-$ generation at these sites [18,19]. In addition to the electron transport chain, mitochondrial ROS may also be generated by pyruvate, $\alpha$-ketoglutarate dehydrogenase, glycerol-3-phosphate dehydrogenase, and monoamine oxidase [20–23]. In apoptotic signaling, release of cytochrome c from mitochondria results in stimulated ROS generation [24]. Under excessive oxidative stress, simultaneous collapse of the mitochondrial membrane potential and a transient increase in ROS...
generation by the electron transfer chain can result in mitochondrial release of ROS to cytosol. This can trigger “ROS-induced ROS release” in neighboring mitochondria [25]. Thus, although a low rate of ROS generation is a normal process in mitochondria, disruption of electron flow with excessive ROS generation can activate cell death.

In addition to ROS, reactive nitrogen species (RNS) including nitric oxide (NO) and peroxynitrite (ONOO$^-$), formed from reaction of NO with O$_2^-$, are present. The interaction of NO with mitochondria is also a potential mechanism for mitochondrial redox regulation. NO at physiological levels in cells does not appear to be directly toxic but can be converted to more reactive species [26]. Similar to ROS reactivity to protein thiols (-SH in cysteine residue), NO is also reactive to protein thiols and glutathione (GSH) to form nitrosothiols [27]. NO-modified proteins or other NO metabolites play a key role in regulating cellular redox signaling [27].

In support of a possible role for NO within mitochondria, mitochondrial nitric oxide synthase (mtNOS) has been identified and its function of NO generation in the mitochondria has been reported [28,29]. In addition to stimulation of mitochondrial NO production by calcium-stimulated mtNOS activation, specific interaction of NO with cytochrome oxidase (complex IV) was shown to affect mitochondrial function [30]. Subsequent research demonstrated that mitochondrial function was affected by interactions of mitochondrial NO and its derivatives with glutathione (GSH) and with protein thiols [31].

NO reacts with O$_2^-$ to produce peroxynitrite at a diffusion controlled rate. Peroxynitrite reacts with tyrosine residues in proteins to form nitrotyrosine residues [32,33]. Similar to NO, ONOO$^-$ has a very short half-life, and its effect on cells is dependent on the concentration and proximity to the target molecule.

The mechanisms of ROS and RNS and their control by free radical scavengers have been extensively reviewed [34–37]. In the present review, we primarily discuss only the thiol/disulfide redox-regulated antioxidant systems. Mitochondria have two major thiol antioxidant systems dependent upon GSH and the small protein thioredoxin-2 (Trx2), and each has several associated proteins, e.g., glutaredoxin-2 (Grx2), glutathione peroxidases 1 and 4 (Gpx1, Gpx4), and thioredoxin reductase-2 (TrxR2) and peroxiredoxins 3 and 5 (Prx3, Prx5), respectively.

### 2.2. NADPH/NADP$^+$ system as reducing power for Trx and GSH system

In contrast to the respiratory electron transport chain in the mitochondria which is dependent upon NADH/NAD$^+$, both Trx and GSH systems are dependent on the reducing power of NADPH/NADP$^+$. Most mitochondrial dehydrogenases are linked to NAD$^+$ rather than NADP$, so the rate of electron flow through the NADH/NAD$^+$ couple is significantly greater than that for the NADPH/NADP$^+$ couple. Despite this, available evidence, which is principally only for rat liver, indicates that the steady-state redox potential of NADPH/NADP$^+$ ($\approx$415 mV) is considerably more negative (reducing) than NADH/NAD$^+$ ($\approx$300 mV) [38]. Mitochondrial NADPH functions in biosynthetic and detoxification reactions, including supply of reducing equivalents to the redox pathways containing GSH and thioredoxins [9]. Earlier studies showed mitochondrial reduction of NADP$^+$ by the energy-linked transhydrogenase [39] as well as a limited number of dehydrogenases [40], but additional studies are needed to allow conclusions concerning the endogenous rates of these reactions in different cell types.

### 2.3. Mitochondrial GSH/GSSG redox state

Estimation of mitochondrial GSH/GSSG redox state is technically challenging due to loss and/or oxidation of GSH/GSSG during isolation of mitochondria. The GSH/GSSG pool has been estimated from GSH and GSSG concentrations following selective permeabilization of cells...
and also from isolated mitochondria incubated with respiratory substrates. Steady-state redox potential (electromotive force, $E_h$) is calculated using the Nernst equation and provides a measure of the tendency of a chemical species to accept or donate electrons. This tendency is quantitatively expressed in volts or millivolts relative to the standard hydrogen electrode reaction ($H_2/2H^+ + 2e^-$). The $E_h$ for an oxidation/reduction couple (e.g., GSH/GSSG) is dependent upon the inherent tendency of the chemical species to accept/donate electrons ($E_0$) and the concentration of the respective acceptors and donors, defined by the Nernst equation (e.g., for the GSH/GSSG couple, $E_h = E_0 + RT/2F \ln ([GSSG]/[GSH]^2)$). The steady-state redox potential, also referred to as the "redox state", of mitochondrial GSH/GSSG, is about $−280$ mV $[24,41]$. This value is more reduced than the cytoplasmic values ($−260$ mV to $−200$ mV) but not as reduced as measured in isolated mouse liver mitochondria ($−330$ mV to $−300$ mV, $[42]$). The latter redox potential values may be more negative due to an artificially high concentration of respiratory substrates, but both cellular and subcellular approaches indicate that mitochondrial GSH/GSSG redox state is more reduced than total cellular GSH/GSSG redox potential.

The GSH/GSSG redox potential in mitochondria is dependent upon concentrations of GSH and GSSG. Because mitochondrial GSH is not synthesized in mitochondria, it must rely on synthesis in the cytoplasm and subsequent transport into mitochondria $[43,44]$. Therefore, GSH transport is a key determinant of GSH/GSSG redox state $[45,46]$. Transport is stimulated by mitochondrial substrates, e.g., malate or pyruvate, and inhibited by excessive glutamate or disruption of the protonmotive force with proton ionophores. Two transporters, the dicarboxylate carrier and 2-oxoglutarate carrier, are major carriers for GSH transport $[46]$. These anion exchange systems use the energy available from the mitochondrial membrane potential to drive the uptake of the anion GSH against the prevailing membrane potential. Over-expression of these two carriers in eukaryotic cells caused increased mitochondrial GSH uptake and protected from oxidant-induced apoptosis $[47,48]$. In addition to the presence of specific carriers, the mitochondrial inner membrane, the prevailing membrane potential, and the pH gradient from cytoplasm to mitochondrial matrix are important factors to determine mitochondrial GSH/GSSG redox $[46]$. Animal models for disease, including alcoholic liver disease (ALD) and Parkinson’s disease, provide evidence for the importance of GSH transport and GSH redox potential in the mitochondrial compartment $[49,50]$. These studies show that the mitochondrial GSH/GSSG redox potential is affected by cellular pathological and toxicological conditions $[49,50]$. For instance, the ALD study shows that the mitochondrial GSH/GSSG couple was decreased by up to 60% in ALD, and GSH transport was inhibited in rats with ALD $[49]$. A variety of studies using cell culture models also support the crucial role played by GSH in mitochondria as a protective effect in apoptotic cell death $[51–53]$. In apoptosis, oxidation of mitochondrial GSH/GSSG stimulated cytochrome c release $[52]$, and GSH depletion resulted in increased ROS which was generated from complex III, suggesting a role for GSH in controlling mitochondrial ROS generation.

### 2.4. Mitochondrial GSH-dependent systems

Mitochondria contain several GSH-dependent enzymes including glutathione peroxidase-1 and 4 (Gpx1, Gpx4), glutathione reductase (GR) and glutaredoxin-2 (Grx2) $[54,55]$. Studies involving Gpx1 knockout mice showed that susceptibility to mitochondrial oxidative stress was enhanced by Gpx1 depletion $[56]$. Cell lines derived from Gpx4 knockout mice ($Gpx4^{−/−}$) showed an increased sensitivity to diquat-induced mitochondrial dysfunction and loss of the membrane potential $[57]$, suggesting that both Gpx1 and Gpx4 in mitochondria have a protective role against oxidative stress.
While Grx1 is found in cytoplasm, Grx2 is principally localized in mitochondria and catalyzes the reversible oxidation and glutathionylation (Pr-SSG) of mitochondrial membrane proteins [58]. Molecular biological studies show that Grx2 has a central role in GSH-dependent redox regulation in mitochondria [59]. Over-expression of Grx2 in HeLa cells decreased susceptibility to 2-deoxy-D-glucose-induced apoptosis and prevented loss of cardiolipin, the phospholipids anchoring cytochrome c to the inner mitochondrial membrane [60]. Although evidence in now accumulating for Trx2, see below, most of the evidence to date indicates that GSH-dependent systems, including Gpx1, Gpx4, and Grx2, are essential for protection of mitochondria against oxidative stress.

2.5. Mitochondrial Trx2 redox state and Trx2-dependent system

Trx2 is a member of the Trx family of small redox proteins that contains a conserved thioredoxin active site of Trp-Cys-Gly-Pro-Cys-Lys [61]. Similar to the Trx1 system found in cytoplasm and nucleus, the Trx2 system includes Trx2, thioredoxin reductase-2 (TrxR2), and mitochondrial peroxiredoxin-3 (Prx3) and peroxiredoxin-5 (Prx5) [61–64]. The active site cysteines of Trx2 are maintained in the reduced form by electron transfer from NADPH, catalyzed by TrxR2. Overexpression of Trx2 in mammalian cells, i.e., osteosarcoma 143B cells and HEK-293 cells, protected against t-butylhydroperoxide and etoposide-induced apoptosis and mitochondrial membrane potential elevation [3,65]. More recently, Trx2 overexpression in vascular endothelial cells showed that Trx2 plays an important role in vascular relaxation by NO [66]. Depletion of Trx2 in a chicken B cell line resulted in ROS increase, cytochrome c release, and apoptosis [67]. Heterozygous Trx2 deficient mice (Trx2+/−), did not have gross phenotype changes while Trx2−/− was embryonic lethal with massive apoptosis [68].

Because Trx2 is specific to the mitochondria, redox western blot analysis of Trx2 has been useful for the selective determination of mitochondrial redox potential by the redox Western blot method [5,69]. Based on the redox potential value obtained using this technique, mitochondria are more reduced than other compartments in the cell [2], which is consistent with the data obtained using the redox sensitive green fluorescent protein [70]. The mitochondrial Trx2 has a much more reduced redox value (~330 mV at pH 7.6) than cytoplasmic and nuclear Trx1 (~280 mV to ~300 mV at pH 7.0, [42]). The fraction of Trx2 present in the reduced form is variable in different cell types and under different metabolic conditions, with redox potential normally in the range of ~360 mV to ~340 mV [42]. Increasing evidence that the redox state of mitochondrial Trx2 is distinct from Trx1 in cytoplasm and nucleus is listed in Table 1. The distinct differences of Trx2 and Trx1 in redox sensitivity are shown by different treatments, including metal ion (arsenic), restriction of glucose (Glc) and glutamine (Gln), and exposure to an inflammatory cytokine (TNF-α) (Fig 1) [69,71,72].

2.6. Thioredoxin reductase-2

Thioredoxin reductases (TrxR) are NADPH-dependent members of the nucleotide-disulfide oxidoreductase family which function to reduce thioredoxins. TrxR's are ubiquitously found in mammalian tissues, are homodimeric and contain a C-terminal selenocysteine necessary for catalytic activity. Three isoforms have been identified, the cytosolic TrxR1, mitochondrial TrxR2, and testis-specific Trx and GSSH reductase [73]. Cytosolic TrxR1 and mitochondrial TrxR2 function independently [74,75], and elimination of either TrxR1 or TrxR2 gene results in an embryonic lethal phenotype [76,77]. TrxR2−/− fibroblasts were much more sensitive to GSH deprivation induced-oxidative stress than TrxR2+/+ [76]. The phenotype of the mice after inactivation of TrxR2 was less severe than that in Trx2 knockout mice [78], which may suggest that other systems including TrxR1 or GSH-dependent redox systems substitute for the TrxR2 deficiency in mitochondria.

Biochim Biophys Acta. Author manuscript; available in PMC 2009 November 1.
2.7. Mitochondrial peroxiredoxins

The mitochondria also contain peroxiredoxins (Prx), members of a super-family of selenium-free and heme-free peroxidases which function as Trx peroxidases to catalyze the reduction of \( \text{H}_2\text{O}_2 \), alkyl hydroperoxide, and peroxynitrite [79]. In mammals, six different peroxiredoxins (Prxs) have been identified. Prx3 is exclusively localized in mitochondria, and Prx5 has been found in mitochondria [80,81] and other compartments, e.g., peroxisomes, cytosol and nucleus [82]. Banmeyer et al. [82] showed that Prx5 protected mitochondrial DNA from oxidative attack, suggesting an important role for Prx5 in mitochondrial genome stability. Prx3 decreased cell growth by regulating \( \text{H}_2\text{O}_2 \) level and protected cells from peroxide-induced apoptosis [68]. Similar to the results from the functional studies for Trx2 and TrxR2, the results from over-expression and depletion studies for mitochondrial Prxs suggest that Prx3 and Prx5 play an important role in mitochondrial redox regulation by functioning as antioxidants [66,83].

3. Nuclear redox regulation

3.1. Nuclear protective functions of GSH and Trx1

ROS and RNS serve as messengers in cell signaling but also cause oxidative damage to macromolecules, including DNA damage. Sequestration of DNA within nuclei protects the genome from reactive chemicals generated in other parts of the cells; however, \( \text{H}_2\text{O}_2 \), organic hydroperoxides, and NO can diffuse across membranes, thus posing a potential damage from normal signaling events. Similar to other cellular organelles, nuclei also contain two major antioxidant systems dependent upon GSH and Trx1.

Studies to evaluate nuclear compartmentalization of GSH are contradictory due to artifacts resulting from perturbations during measurement [84–87]. However, an indirect estimation of nuclear GSH/GSSG, can be obtained by measuring nuclear S-glutathionylated protein (Pr-SSG), which is less subject to artifactual redistribution. Pr-SSG can be formed from reaction of GSSG with protein thiols (PrSH) and reaction of oxidized proteins with GSH. In principle, PrSSG could also be formed by GSH S-transferases or thiol peroxidases. Pr-SSG concentrations in nuclear and cytoplasmic compartments can be measured following reduction of nuclear and cytoplasmic proteins fractions with DTT, releasing GSH for HPLC measurement [88]. Results from this approach showed that cytoplasmic proteins have a higher content of S-glutathionylation than nuclear proteins. Stimulated nuclear production of \( \text{H}_2\text{O}_2 \) by targeting D-amino acid oxidase to nuclei resulted in increased protein glutathionylation in the nuclei but not in the cytosol [89]. In contrast, culture of cells in media devoid of Glc and Gln resulted in increased protein S-glutathionylation in the cytosol but not in nuclei [71]. Thus, the data show that protein S-glutathionylation is independently controlled in the cytoplasmic and nuclear compartments and indicate that the GSH/GSSG redox potential is likely to be more reduced in nuclei than in cytoplasm.

Compared to the GSH/GSSG redox measurement, measuring Trx1 redox in nuclei is less challenging. Trx-1 can translocate into the nucleus in response to a variety of stimuli including oxidants, UV irradiation, cytokines, lipopolysaccharide [90,91], and Trx1 in nuclei has been shown in both cell culture and animal models [92,93]. The reason for the increase in nuclear Trx1 in response to stress has not been established; however, it may be due to its antioxidant and repair functions against oxidant-induced damage. Alternatively, the increase in nuclear Trx1 concentration may provide a more reducing environment required for DNA binding by transcription factors (see below). Transport has recently been shown to be dependent upon importin-\( \alpha \) [94], and retention could be maintained by binding to thoredoxin binding protein-2 (TBP-2), a nuclear protein also identified as Vitamin D\textsubscript{3}-binding protein-1 [95].

*Biochim Biophys Acta. Author manuscript; available in PMC 2009 November 1.*
3.2. Nuclear Trx1 is functionally autonomous from cytoplasmic Trx1

A distinct nuclear pool of Trx1 is evident from immunohistochemical studies demonstrating that Trx1 is imported into the nucleus during oxidative stress [90,94,96,97]. Although studies by Schroeder showed that lysine residues K81 and K82 of Trx1 play a key role in nuclear transport [94], the detailed mechanisms for nuclear localization of Trx1 remain to be identified. The existence of Trx1 in both compartments, cytoplasm and nucleus, provides the basis for its use in the study of nuclear and cytoplasmic redox states. Several lines of evidence now indicate that the redox state of Trx1 in the nucleus is distinct from that in the cytoplasm (see Table 2 and Fig. 2). Quantitative measurements show that reduced:oxidized ratios for Trx1 differ by 5- to 6-fold under basal conditions in different cell lines [2].

3.3. Cysteine (Cys)-containing proteins and GSH in cell nuclei are critical for cell function

Cys-containing proteins control the genome, and include enzymes, transport machinery, structural proteins and transcription factors with conserved cysteine in zinc fingers and DNA-binding domains. Many of these nuclear systems depend upon the abundant, low molecular weight thiol antioxidant, GSH. GSH is important in the regulation of the nuclear matrix organization [98], maintenance of Cys residues on zinc-finger DNA binding motifs in a reduced and functional state [99]; chromosome consolidation [100]; DNA synthesis [101]; DNA protection from oxidative stress [102] and protection of DNA-binding proteins [103]. GSH supports GSH S-transferases (GST) in protection against oxidative DNA damage [104]. GSTpi in nuclei is increased in some cancers and is associated with anticancer drug resistance [105, 106]. Moreover, GSTpi forms a complex with peroxiredoxins [107] and catalyzes S-glutathionylation of Prx5 [108].

3.4. Redox-dependent activation of transcription factors by GSH and Trx1 systems

Regulation of transcription factor activity is among the most actively studied and important aspects of nuclear redox control. Several important transcription factors, including AP-1, NF-kB, Nrf2, p53 and glucocorticoid receptor [109–114] contain a critical Cys in the DNA binding region which is required for DNA binding. Accumulating evidence indicates that both GSH and Trx1 are important in regulation of transcription factor activity, apparently by different mechanisms.

3.4.1. Activating protein-1 (AP-1) activation—Early studies of activator protein-1 (AP-1) showed that oxidative stress signals phosphorylation of Jun to activate AP-1 [115–117]. In contrast, AP-1 binding to DNA is inhibited by high oxidant conditions due to a redox-sensitive thiol in the DNA binding region [109,118]. Redox factor-1 (Ref-1) restores DNA binding using Trx1 as a reductant [119,120]. Thus, AP-1 has two opposing redox-sensitive steps, 1) an upstream oxidative activation of the signaling kinase pathway and 2) a downstream Trx1-dependent reduction of the Cys of the DNA-binding domain. In addition, an indirect effect of Trx1 in cell nuclei can occur due to Trx1-dependent peroxide metabolism through peroxiredoxins (Prx1, Prx2, Prx5)[121].

3.4.2. Nuclear factor-κB (NF-κB) activation—Similar opposing responses in cytoplasm and nuclei also occur for NF-κB. NF-κB activation in the cytoplasm is initiated by I-κB kinase, which phosphorylates I-κB causing dissociation and release of NF-κB for translocation into the nucleus [122]. Many activators of NF-κB, such as TNF-α and phorbol esters, stimulate ROS production, and activation can be blocked by reducing agents [113,123] showing that activation of NF-κB is regulated by redox-dependent mechanisms. Over-expression of Trx2 in mitochondria blocked TNF-α-dependent activation, indicating that mitochondrial ROS can function to activate in the cytoplasm [124]. However, excessive ROS production, as well as NO generation by SNP (sodium nitroprusside) and GSNO (S-nitrosothiol), modifies a
critical Cys$^{62}$ in the DNA binding region of NF-κB p50 and inhibits DNA binding [125–127]. Thus, as with AP-1, there is a dual effect of ROS and RNS on NF-κB activity. Transfection with Trx1, which is principally present in cytoplasm, inhibited NF-κB activation, but nuclear-targeted Trx1 enhanced DNA binding [90]. As with AP-1, this process is mediated by Ref-1 [128,129]. Targeted expression of a nuclear export signal-containing Prx-1 in the cytoplasm resulted in inhibited NF-κB reporter activity while the corresponding construct with a nuclear localization signal stimulated activity [124]. This experiment indicated that Prx-1-dependent H$_2$O$_2$ metabolism in the nucleus is important in controlling the NF-κB reporter activity [124]. However, in contrast to this, direct stimulation of H$_2$O$_2$ production in nuclei by targeted increase in D-amino acid oxidase resulted in inhibition of a NF-κB reporter activity but occurred without Trx1 oxidation [89]. The latter inhibition of NF-κB reporter activity was associated with increased nuclear protein S-glutathionylation. Thus, even though Trx1 provides a primary redox regulation of NF-κB in nuclei, this system also appears to be controlled by the GSH system [89].

3.4.3. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activation—Control of Nrf2 is similarly complex [130,131], with kinase control mechanisms as well as redox control [132]. Similar to Fos, Jun and NF-κB, Nrf2 has a critical Cys$^{508}$, which must be in the reduced form to bind to DNA [111]. Oxidation of Nrf2 blocks its ability to increase gene expression. As shown with AP-1 and NF-κB, Trx1 reduces Cys$^{508}$ in a Nrf2-dependent reaction and restores gene expression [111]. Recent studies further support compartment-specific redox control of Nrf-2 signaling. Cytoplasmic activation of Nrf-2 was regulated by GSH and not by Trx1 [133]. However, nuclear expression of an ARE reporter was controlled by nuclear-targeted Trx1 and not by GSH [133]. Thus, the GSH and Trx1 systems have distinct functions in the two compartments in control of transcription by Nrf2.

4. Cytoplasmic redox regulation is different from that in nuclear compartment

Redox regulation, like phosphorylation, is a covalent regulatory system that controls normal cellular functions of cells and organisms. It controls how cells respond to stress involving oxidants and free radicals, which underlie many degenerative diseases, including heart disease, cancer, stroke, diabetes, Alzheimer’s disease, and kidney disease. Growth factors, such as epidermal growth factor (EGF), bind to their receptors and stimulate tyrosine kinase activity, initiating a phosphorylation cascade that activates DNA replication and cell division as downstream effects [134]. An increase in ROS accompanies EGF binding to its receptor, which appears to be a critical component for proper signal transduction. Recent studies on EGF stimulation evaluated ROS production, phosphorylation of the receptor, and oxidation of redox couples in different subcellular compartments [5]. Under this system, nuclear Trx1, mitochondrial Trx2, and cellular GSH did not show any significant oxidation following EGF stimulation while cytoplasmic Trx1 was oxidized by nearly 20 mV [5]. Although the effects of specific cytoplasmic oxidation of Trx1 are not known, these findings demonstrated that physiologic stimulation at the plasma membrane can cause the oxidation of specific redox couples in the cytoplasm without affecting other subcellular compartments.

4.1. Trx1 and GSH/GSSG redox couples in cytoplasm are not in equilibrium

The redox state of cytoplasm has been measured using cytosolic Trx1 and GSH/GSSG. The total cellular GSH/GSSG couple largely represents the cytoplasmic GSH/GSSG pool. However, the total cellular GSH/GSSG redox potential is more reduced in cultured cells and tissues in vivo than values obtained for red blood cells, where there are no intracellular organelles. In red blood cells, the E$^\text{h}$ value for GSH/GSSG was estimated to be $\sim$193 mV [135]. This value is more positive (oxidized) than estimates for cells with nuclei and mitochondria, which are typically lower than $\sim$200 mV, indicating that organelles with more
negative value could introduce an error when using a cellular value to estimate the cytoplasm value. Alternatively, the content of disulfide forms in the secretory pathway could result in total cellular values being more oxidized than cytoplasmic values. The magnitude of the latter error was estimated to be about 10 mV for the Cys/CySS couple from digitonin fractionation studies [136].

The standard potential ($E_0$) of the active site dithiol/disulfide couple of Trx1 was estimated to be $-230$ mV [4]. Using this value and measuring the percentage reduction with a redox western blot [4], cellular Trx1 has a steady-state redox potential of $-280$ mV. The value was more negative (reduced) than the GSH/GSSG redox couple, and the couples were not in redox equilibrium (see Fig. 3) [5,133,137]. The Trx1 and GSH/GSSG couples were found to vary independently during growth transitions [137], redox signaling [5,133], and metal-induced toxicity [69]. Additional results by Hansen et al. show that GSH and Trx1 systems have unique functions in Nrf2 activation in the cytoplasm supporting differential roles of Trx1 and GSH in activation of transcription factor in cytoplasm [133]. Thus, the data show that the major redox systems consisting of Trx1 and GSH are distinct both between and within compartments.

5. Extracellular redox state

5.1. Cys/CySS couple is the major low molecular weight thiol/disulfide redox couple in most extracellular compartments

Cys and its disulfide, cystine (CySS) pool constitute the major low-molecular weight thiol/disulfide couple in mammalian plasma [138], and the Cys/CySS pool is one of the central redox control nodes in the biological signaling [136]. As described earlier [2,9,139,140], the redox state of Cys/CySS in the extracellular compartment plays a major role in redox communication between cells and tissues. This function is in contrast to GSH in extracellular fluids, which appears to principally function in detoxification of reactive oxidants in the lung [141] and electrophiles in the small intestine [135]. Although we focus here on plasma Cys/CySS because cells in culture regulate the redox potential in the culture media to values similar to plasma Cys/CySS [142,143], $E_0$ data for plasma GSH/GSSG are also available and show that the redox potential of GSH/GSSG also correlates with health parameters [135,144,145]. Albumin also contains a redox-active Cys residue which is normally about 50% present as the S-cysteinyl disulfide form. This thiol has been suggested to function as a carrier of NO and also to function in detoxification [146,147]. The content of this disulfide was also found to correlate with the plasma Cys/CySS redox potential [138]. Smaller amounts of S-GSH and S-cysteinylglycine disulfides were also present, but these did not correlate with plasma GSH/GSSG redox potential [138], consistent with earlier radiotracer studies which showed that S-cysteinylation is much more rapid than S-glutathionylation in plasma [88]. Trx1 is also present in plasma principally in the oxidized state [148].

The mean plasma GSH/GSSG redox value is approximately $-140$ mV in young healthy individuals [138]. This value is 90 mV more oxidized than the cytoplasmic pool; the difference is roughly equivalent in energetic terms to the membrane potential across the plasma membrane in an electrically excited tissue. The mean plasma Cys/CySS redox state is approximately $-80$ mV, about 60 mV more oxidized than cytoplasmic Cys/CySS redox. Particularly noteworthy, the plasma Cys/CySS redox is not in equilibrium with the plasma GSH/GSSG pool. A recent study of the diurnal variation of these pools showed that the Cys/CySS redox state becomes reduced after meals, with maximal values 2–3 h after eating [149]. GSH/GSSG also became more reduced, but this occurred 6–7 h after maximal changes in Cys/CySS [149]. Other studies show that the plasma redox potentials are oxidized in association with age [138,150], but with different characteristics. GSH/GSSG is unchanged until about 45 years, then becomes oxidized.
at a rate of 0.7 mV/year; whereas, Cys/CySS becomes oxidized by about 0.2 mV/year over the entire age range from 18 to 93 years [138]. In addition to aging, plasma thiol/disulfide redox state is oxidized with oxidative stress involved circumstances such as chemotherapy [145], diabetes [135], cardiovascular disease [144], and smoking [151]. Alcohol abuse has recently been shown to result in oxidation of the redox potential in the lung lining fluid [141]. The lining fluid has a high GSH concentration and the GSH/GSSG redox state is similar to tissue redox values, i.e., −200 mV, perhaps reflecting the functional need to protect against oxidative toxicity and/or to avoid formation of protein disulfide. Fluidity of mucus is decreased by an oxidized redox state [152,153], and crosslinking of proteins on the cell surface or in the lining fluid could also occur due to oxidation of the redox state. Thus, maintenance of a highly reduced redox state in the lining fluid would appear to be necessary to maintain deformability of the alveolar structures as well as signaling and other homeostatic functions. The plasma redox value correlates with plasma protein S-glutathionylation [154,155], and age-associated oxidation of plasma redox couple can be prevented by antioxidant supplements [156].

5.2. The effects of changes in extracellular Cys/CySS redox on intracellular signaling

Recent advances have been made in understanding the mechanisms and functional consequences of redox changes in the extracellular compartment, as summarized in Table 3. These studies have addressed the potential consequences of variation in plasma Cys/CySS redox state by systematically varying this parameter in cell culture. In human gut epithelial (Caco-2) cells and normal human retinal pigment epithelial (hRPE) cells, cell proliferation was greater at more reduced $E_h$ values (Fig. 4, [143,157]). In studies to identify the mechanism of extracellular Cys/CySS redox state regulated cell growth in Caco-2 cells, alteration of the extracellular Cys/CySS redox state was sufficient to alter phosphorylation of p44/p42 MAPK through EGFR phosphorylation, with the greatest phosphorylation observed at the most reducing conditions (−150 mV) [158]. On the other hand, a lung fibroblast model relevant to pulmonary fibrosis showed that oxidized $E_h$ values stimulate fibroblast proliferation and matrix expression through upregulation of transforming growth factor-β (Fig. 4, [159]). These results suggest that extracellular Cys/CySS redox-dependent cell proliferation is cell type specific and is mediated by intracellular kinase (e.g. p44/p42 MAPK) activation.

The cell type specificity may reflect differences in cell responses to physiologic conditions, wherein fibroblasts respond to proliferate for tissue repair following immune cell-induced oxidative conditions, while reducing conditions are most stimulatory to other cells. Importantly, many studies show that there is growth stimulation both due to low levels of ROS and in association with increased cellular GSH and/or a more reduced extracellular thiol/disulfide redox state [157]. This apparent contradiction occurs because thiol/disulfide changes affect an upstream redox signaling step while the ROS affects a more downstream (intermediate) signaling step. Activation of these signaling molecules-dependent cell growth and proliferation mechanisms has been shown in a broad range of eukaryotic cells including cancer-derived and primary cells [158–161] and these mechanisms have been shown redox sensitive [162,163].

Although there are relatively small numbers of studies available on extracellular Cys/CySS redox-dependent changes, the available data show that cell growth control is mediated by intracellular kinase activation [142,158,159]. The different responses among cell types, however, indicate that underlying mechanisms of signaling in response to extracellular Cys/CySS redox controlled cell growth and proliferation will require further elucidation.

While proliferation rate of hRPE cells were greater at reduced extracellular condition, these cells were more sensitive to oxidant-induced apoptosis than at more oxidized $E_h$ values (Fig. 4, [143]). Additional studies to examine whether extracellular Cys/CySS redox state controls vascular biology using cultured vascular endothelial cells and human THP1 monocytes showed

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that ROS production was increased in cells exposed to a more oxidized Eh value ([142]). Also, increased numbers of adherent THP1 cells were observed when endothelial cells where exposed to culture medium containing oxidized Cys/CySS Eh (Fig. 4 [142]). Thus, the data show that the factors controlling the balance of plasma or extracellular Cys/CySS are important in determining cell functions, with general characteristics that a more reduced state stimulates cell proliferation while a more oxidized state sensitizes cells to oxidative stress and other stress responses.

5.3. Trx1 in extracellular compartments

Within the cytoplasm, nucleus, and mitochondria, Trx1 regulates redox signaling molecules and antioxidant functions as described in earlier sections of this review. In addition to translocation into the nucleus, Trx1 is secreted to the extracellular compartment where it can serve as a biomarker of disease and also can exert important protective functions [164–166]. Trx1 found in the extracellular compartment exists in two forms including intact Trx1 (12 kD) and a truncated form (10 kD). Intact Trx1 was found predominantly in an oxidized form [148,167] and its truncated form (Trx-80, 10 kD) was found containing only N-terminal 1–80 (or 1–84) residues of Trx1. Truncated Trx1 can be released by cells [168] and functions with both cytokine-like and chemokine-like activities [169,170]. Extracellular Trx1 prevents LPS- and chemokine-induced neutrophil chemotaxis in mice [165] and decreases reperfusion-induced arhythmias [171] and adriamycin-induced cytotoxic injury [172]. Extracellular Trx1 suppresses autoimmune myocarditis via its antioxidative damage and anti-inflammatory functions [173] and prevents ischemic reperfusion injury [174]. Although the mechanism that mediates secretion of Trx1 to extracellular compartment and related effects need to be elucidated, Trx1 secretion occurs predominantly under conditions associated with oxidative stress, apoptosis, and inflammation [148,167]. Consistently, plasma Trx1 concentrations were raised in patients with several cardiovascular risk factors [175] and with acute lung injury [176]. Hoshino et al. suggested that secreted Trx1 in extracellular compartment, plasma/serum Trx1, can be a useful biomarker for a wide variety of diseases.

6. Peroxisomes

Peroxisomes are ubiquitous organelles that perform biochemical functions of lipid metabolism and a number of oxidative reactions involving H₂O₂. The activities in mammalian peroxisomes include α-oxidation of specific fatty acids, catabolism of purines, polyamines, and prostaglandins, the biosynthesis of plasmalogens and sterols, and regulation of ROS and NO metabolism [177]. Peroxisomal enzymes generating ROS, primarily H₂O₂, were summarized in recent reviews [178,179]. ROS generating enzymes include acyl-CoA oxidase, urate oxidase, xanthine oxidase, D-amino acid oxidase, pipecolic acid oxidase, D-aspartate oxidase, sarcosine oxidase, glycolate oxidase, hydroxyacid oxidase 2 and 3, and polyamine oxidase [178,179]. NO production in peroxisomes has been shown and NO generation was regulated by inducible nitric oxide synthase detected in peroxisomes [180]. Therefore, it is clear that peroxisomes are another source of oxidants, ROS and NO, due to the presence of these enzymes besides mitochondria.

Earlier studies to evaluate H₂O₂ metabolism in rat hepatocytes showed that at low rates of generation by glycolate oxidase, most of the peroxide was metabolized by catalase within the peroxisomes [181]. However, at rates of generation >6 nmol/10⁶ cells per min, H₂O₂ generation was sufficient to cause loss of cellular GSH, indicating that GSH-dependent metabolism was stimulated [181]. Peroxisomal H₂O₂ concentrations under these conditions were approximately 40 nM. In contrast, stimulation of H₂O₂ production in the endoplasmic reticulum resulted in substantial GSH loss with less peroxisomal H₂O₂. These data show that at low rates of production, peroxisomal and non-peroxisomal H₂O₂ metabolism are largely separated according to the respective sites of generation [181]. Steady-state infusion of

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H$_2$O$_2$ under anaerobic conditions allowed calibration of catalase and GSH peroxidase activities (without consideration of peroxiredoxin contribution) [182]. Results showed that non-catalase dependent metabolism represented a substantial fraction of the total elimination at low rates of infusion. However, at high rates, H$_2$O$_2$ was nearly stoichiometrically converted to O$_2$ according to the catalatic reaction [182], indicating that the compartmentalization of H$_2$O$_2$ metabolism under physiologic conditions is due to rapid kinetics compared to diffusion.

Eukaryotic peroxisomes also contain antioxidants in addition to catalase, including glutathione peroxidase (Gpx), manganese superoxide dismutase (SOD2), copper/zinc superoxide dismutase (SOD1), epoxide hydrolase, peroxiredoxin-1 (Prx1), and Prx5 (also known as PMP20) [179]. However, as indicated above, catalase is the major peroxisomal enzyme responsible for the metabolism of H$_2$O$_2$. Inhibition and mislocation of catalase resulted in multiple peroxisomal enzymatic dysfunctions and deficiencies [183,184]. Deficiency of peroxisomal catalase caused developmental abnormalities while its overexpression in transgenic mice resulted in extended life span suggesting that catalase in peroxisomes has an important role in cell survival and aging mechanisms [185].

While numerous studies have focused on catalase and its function, relatively little is known about other antioxidant enzymes, such as Gpx and Prxs, and their roles in mammalian peroxisomes, even though these enzymes are important in plant peroxisomes [186]. Since these enzymes are members of the GSH/GSSG and Trx systems, additional studies in mammalian peroxisomes are needed to evaluate their functions and also to estimate peroxisomal redox state.

7. Endoplasmic reticulum (ER)

While mitochondria can be generalized as a compartment utilizing oxidation for energy production and the peroxisomes as one utilizing H$_2$O$_2$ as an oxidative reagent, the ER is specialized with different oxidative functions in different cell types. In liver and steroidogenic cells, ER contains hemoproteins of the cytochrome P450 (Cyp) family which function to activate O$_2$ to a reagent form used for steroid synthesis and oxidative detoxification of foreign compounds [187]. Many of these are highly inducible. The ER, as well as many other cell membranes, contains high amounts of cytochrome b$_5$, a component of stearyl-CoA desaturase and a number of other oxidase enzymes [188]. Each of these hemoprotein systems is associated with flavoprotein reductases, and these systems can generate considerable amounts of O$_2$ and H$_2$O$_2$ under some conditions. In muscle, redox reactions function in regulation of Ca$^{2+}$ signaling through the ryanodine receptor [189].

In addition to these more specialized oxidative functions, the ER has a more general oxidative function to introduce structural disulfides in protein folding for membranal and exported proteins [190]. Central redox proteins for these functions include the endoplasmic reticulum oxidase-1 (Ero-1) and protein disulfide isomerases (PDI). Ero-1 proteins are flavoproteins which are oxidized by O$_2$ and in turn oxidize dithiols in PDI to disulfides. Ero-1α is a constitutive form while Ero-1β is an inducible form produced under conditions of increased secretory demand or when processing results in excessive improperly folded protein, termed the unfolded protein response [191]. Ero-1p contains two catalytically inactive cysteines which serve as a built-in “off” mechanism, becoming oxidized and inactivating the enzyme under excessively oxidizing conditions [191]. Many PDI homologs function in the folding and processing pathways in the ER, and the appropriate function of these proteins requires a dynamic condition of partial reduction in the cyclic process of introduction of disulfides. Exposed reactive cysteines of processed proteins serve as retention signals within the ER [192] and a critical Cys29 of ERp44, a protein located more distally in the secretory pathway, functions in quality control for the proper folding [193].
In addition to the oxidative mechanisms [194], ER also contains a reductase system regulated by GSH transported by isolated microsomes [195]. GSH in the ER is used to reduce incorrect proteins disulfides. In the process, GSH content is depleted and this diminishes the capacity of the ER to counteract ROS [196]. Thus, ER contains both oxidative and reductive mechanisms which allow dynamic control of redox state in association with processing and secretory functions [197].

The GSH/GSSG ratio in the ER varies between 3/1 and 1/1 and is considerably more oxidized than cytoplasmic GSH/GSSG ratio (30/1 to 100/1). Based upon these values the ER redox potential has been estimated to be $-189$ mV, which is relatively oxidizing compared to other cellular compartments [198]. A significant amount of GSH in the ER was detected as protein mixed disulfides, which appears to regulate the activity of redox-active thiol-containing proteins and protect against hyperoxidizing conditions [196]. Interestingly, in an animal model for study of elastase regulation in lung function, the ER redox state in α1-antitrypsin transgenic mice was more reduced by expression of α1-antitrypsin, and this was accompanied with a higher GSH/GSSG ratio compared to the cytoplasm. The results indicated that changes in ER redox state to a more reduced environment resulted in a more protective role of ER against oxidative stress [199].

8. Lysosomes

Conditions for degradation of proteins to small peptides in lysosomes include an acidic pH and reduction of inter- and intrachain disulfide bonds. Due to the low pH, disulfide reduction in this compartment may not be favored by Trx or GSH redox systems that normally act in a neutral environment. There has been no enzyme found to catalyze reduction of the disulfide bond besides gamma-interferon (IFN-γ)-inducible lysosomal thiol reductase, GILT [200]. GILT, a unique lysosomal thiol reductase induced by IFN-γ, contains an active site with two cysteines like Trx family members and shows disulfide reduction activity at acidic pH. The redox potential of lysosomes has been measured in PC3 cells by expressing a redox-sensitive variant of GFP fused to various endocytic proteins (roGFP) [70]. The results showed that lysosomal redox potential was at least as oxidized as $-240$ mV, while mitochondrial redox potential was measured as $-318$ mV using roGFP, suggesting that lysosomes are relatively oxidized compared to other subcellular organelles [70]. In addition, deficiency in mechanisms to reduce disulfides in lysosomes was also shown by earlier studies in cystinosis, where a mutation results in loss of CySS transport and an accumulation of CySS within lysosomes [201,202].

Non-thiol-dependent mechanisms also exist in the secretory pathway, and investigation of the interaction of these systems with thiol/disulfide systems is needed. Coenzyme Q, ubiquinone (UQ) is widely distributed in cell membranes and could play a role in antioxidant function and proton transport in other membranes including plasma membrane, golgi vesicles, and lysosomes [203,204]. In lysosomes, a redox chain exists which carries electrons in the sequence of NADH > FAD > cytochrome b > UQ > oxygen, and regulates acidification of the lysosomal matrix. High levels of UQ exist in the reduced state in lysosomes and this contributes to distribute protons and to ROS generation [204,205]. On the other hand, reduced UQ restores antioxidant function to oxidized tocopherol while no enzymes are available for reduction of oxidized tocopherol, directly suggesting that UQ has a dual function, antioxidant and oxidant, in lysosomal redox cycling systems.

9. Endocytic pathway

In opposition to secretory mechanisms, uptake and activation of toxins, such as diphtheria toxin, cholera toxin and Pseudomonas exotoxin, through the endocytic pathway involves reduction of disulfides [206–209]. Studies with a disulfide-containing radiolabeled polymer
showed that the process in Chinese hamster ovary cells was partially blocked by thiol reagents and occurred over a several hour time course [210]. The process was inhibited by antibody to PDI, indicating that cell surface PDI was involved in the process, but the several hour time course indicated that intracellular reduction also occurred. The dynamics of this process have been studied with FRET using a folate-BODIPY disulfide with rhodamine which allowed real-time imaging of reduction in killer butterfly (KB) cells [8]. Results showed that reduction occurred with a half-life of 6 h, began in endosomes, did not depend significantly on extracellular surface thiols or redox machinery within lysosomes or Golgi, and yielded products which were sorted into different endosomal pathways. Thus, cells possess pathways to regulate the redox gradient of thiol/disulfide components in both release and uptake directions.

10. Summary and Perspectives

The available evidence shows that the concept of a simple redox balance within biologic systems is an inadequate description because each compartment of cells has different redox characteristics and, within each compartment, the major thiol/disulfide control systems are not in thermodynamic equilibrium. The redox characteristics of the compartments appear to have evolved in conjunction with the functions of the organelles, with mitochondria optimized for high-flux electron transfer reactions which are well insulated from other redox processes both within mitochondria and in other parts of the cell. Redox communication between the mitochondria and other compartments occurs via small reactive species, eg $O_2^-/H_2O_2$, but the quantification and specificity of this communication mechanism requires further clarification. The nuclear compartment is relatively reducing and resistant to oxidative stress, perhaps reflecting a function of oxidative reactions as an off mechanism for central transcription factors. The secretory pathway and endocytic pathway have opposing redox systems which respectively oxidize and reduce thiol/disulfide couples in association with movement out or in. Because of the critical nature of folding, the secretory pathway contains quality control mechanisms dependent upon thiol/disulfide state. The very high rates of oxidation required for cells with high secretory function appear to require this compartmentalization to preserve a more reduced state in other regions of the cell. The cytoplasm is relatively devoid of enzymes which generate reactive oxygen species as part of normal metabolic function. This can serve two purposes. First, it can provide a buffer zone between mitochondria, ER and nuclei, so that each organelle is protected against aberrant ROS production by the others. Second, it can allow a very low background of ROS and RNS so that these can be used for sensitive and specific signaling dependent upon NADPH oxidases and NO synthases [211]. Finally, the extracellular compartments provide defensive barriers against external oxidants, a medium for communication between cells and appropriate conditions for receptor and transporter functions in the plasma membranes. Redox state measurements within the extracellular compartments are emerging as a useful biomarker of disease risk and provide a means to both monitor disease progress and response to interventions.

Detection methods with sufficient sensitivity, spatial resolution and chemical/biochemical specificity remain a major limit to progress in understanding redox compartmentalization. However, rapid advances with genetically engineered redox-sensitive GFP’s as well as fluorescent probes and new nanosensors appear likely to overcome these limitations. Much of what is known has been obtained with cell lines under a very limited range of conditions. One can expect that evolution of highly differentiated functions of organs and tissues will have resulted in associated variations which are critical to function of specific cell types. Thus, while the general features of subcellular redox compartmentalization have been elucidated, there remain many unanswered questions concerning the details in different biologic and pathologic conditions.
The thermodynamic disequilibrium of redox couples within and between compartments was recently considered from the perspective of redox systems biology [42]. A central principle is that electron transfer rates between thiol/disulfide systems are slow under prevailing conditions in cells. Key aspects for systems biological descriptions are summarized in Fig 5. Insulation of different redox components occurs by compartmentalization and also by organization of macromolecular complexes. Redox communication between compartments and complexes is poorly understood and represents an important subject for investigation. Within compartments and complexes, definition of pathways remains uncertain because of the differences in rates of thiol/disulfide reactions. In principle, this subject can be clarified by more specific consideration of proteins in terms of redox control by thioredoxin and GSH-dependent systems. Apoptosis signal-regulated kinase-1 (Ask-1), Nrf2 and NF-κB are controlled differently by Trx1 and GSH [89,133,212]. Additional studies are needed to understand these mechanisms, which appear to be orthogonal redox control mechanisms operating through different redox-sensitive elements.

Finally, there is considerable need to integrate these concepts of thiol/disulfide compartmentalization into the broader context of the biologic consequences of redox signaling. Enhanced generation of oxidants is a common feature of cell proliferation, and the coordinated oxidant generation with changes in thiol/disulfide redox status may be an important aspect of the context-dependent regulation of cell function [213,214]. This subject has been extensively studied and is relevant to neurotrophin signaling in neurons [215] and to oncogenic signaling by H Ras [216–218]. A similar connection occurs between oxidative stress in mitochondria and the stimulation of apoptosis or senescence [219,220]. At the molecular level, these aspects are integrated by diffusible ROS species, which provide communication between compartments. For instance, mitochondria-derived H$_2$O$_2$ provides a transmembranal redox signal for cytoplasmic activation of c-Jun N-terminal kinase through Ask-1 [221]. A review on the specificity of ROS in signaling has recently become available [211] and provides many additional examples and details of these mechanisms. Additional investigation of the cell biology of the ROS and kinase signaling mechanisms within the developing knowledge of compartmentalization of thiol/disulfide redox systems provides an opportunity to improve understanding of these critical pathways in disease.

Acknowledgements

Research in the authors’ laboratory, upon which a portion of this review is based, was supported by NIH Grants ES009047 and ES011195.

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Figure 1.
Mitochondrial Trx2 is more sensitive to oxidants than Trx1. Cells were exposed to arsenic (As), glucose and glutamine depletion (-Glc-Gln), or TNF-α. To provide reduced and oxidized controls, cells were treated with DTT (5 mM) and H₂O₂ (2 mM), respectively. Redox analyses of Trx2 and Trx1 were obtained by redox western blot methods [4,5]. Briefly, the separation of Trx2 redox forms was obtained by difference in molecular weight due to alkylation of thiol in reduced Trx2 with AMS (4-acetoamido-4-maleimidylstilbene-2,2-disulphonic acid, 500 Da) under non-reduced condition of SDS-PAGE (Fig. 1, top). For Trx1 redox analysis, thiol alkylation of reduced Trx1 by IAA (iodoacetic acid) introduces extra negative charges, thereby giving rise to faster mobility in reduced form than oxidized form under native condition of PAGE (Fig.1, bottom). Using these methods, the results show that Trx2 with 100 µM As [72], Glc and Gln depletion [71], or TNF-α (20 ng/ml) [69] is significantly more oxidized than Trx1 (Fig. 1).
Figure 2.
Nuclear redox system is distinct from cytoplasm and is protected from oxidation. A. Redox state of Trx1 shows that oxidation of cytosolic but not nuclear Trx1 occurs in Glc- and Gln-free media [71]. B. Cells transfected with vector control or nuclear-targeted Prx1 (NLS-Prx1) for 24 h were exposed to H2O2 for 20 min with indicated amounts. BIAM (biotinylated iodoacetamide) labeling followed by immunoprecipitation for p50 NF-κBα was used to determine reduced p50 NF-κB [124]. NLS-Prx1 expression inhibited oxidation of p50 compared with vector control (Mock). C. Cells were transfected with vector control (Mock) or nuclear targeted-D-amino acid oxidase (NLS-DAO) and treated with N-acetyl-D-alanine (NADA, 0 – 1 mM) to stimulate H2O2 production in the nuclei of cells [89]. NLS-DAO/NADA-induced ROS caused significant oxidation of the nuclear but not the cytosolic GSH pool, as measured by protein-S-glutathionylation (Pr-SSG).
Figure 3.
Independent redox regulation of cellular (cytoplasmic) GSH/GSSG and cytoplasmic Trx1 by EGF, As, and differentiation. Redox states of cellular GSH/GSSG (left, open symbols with solid lines) and Trx1 (right, filled symbols with dashed lines) were measured in cells treated with EGF (circle, [5]) and arsenic (As) (rectangle, [72]), and in cells progressing from proliferation to differentiation (triangle, [137]). EGF treatment caused significant oxidation of cytoplasmic Trx1 redox state (20 mV) but had no effect in GSH/GSSG redox state. Similarly, arsenic oxidized Trx1 but not GSH/GSSG. GSH/GSSG but not Trx1 redox state was significantly oxidized during cellular differentiation.
Figure 4. Extracellular Cys/CySS redox-controlled changes in cellular mechanisms. Cells exposed to Cys/CySS redox-controlled media, varying from most reduced (−150 mV) to most oxidized (0 mV) stimulated monocyte adhesion to endothelial cells (A, [142]), reduced proliferation in colonial epithelial cells, Caco2 (B, [157]), increased cell death due to oxidants (C, [143]), and stimulated fibroblast growth (D, [159]).
Non-equilibrium thermodynamics of biologic redox systems

1. Biology requires both insulation and communication of redox systems
   a. Compartmentalization and organization of macromolecular complexes provides an important mechanism for biologic redox control
   b. Redox communication between compartments/complexes is critical for function; mechanisms are poorly understood

2. Redox pathways within compartments are difficult to define
   a. Thiols and disulfides are abundant
   b. Most thiol/disulfide exchange reactions are slow under prevailing conditions
   c. Relative rates of redox reactions vary considerably; redox pathways are often defined by measured interactions rather than by redox reaction rates
   d. GSH and thioredoxins function as independent redox control nodes, each controlling multiple redox-active proteins

3. Orthogonal redox regulatory mechanisms within pathways
   Individual proteins contain multiple thiol/disulfide redox elements which, in principle, can provide independent redox control mechanisms governing a single biologic process

Figure 5.
Non-equilibrium thermodynamics of biologic redox systems. A recent review of thiol/disulfide systems from the perspective of developing mathematical models for the dynamics of redox regulation identified three important areas for investigation [42]. The insulation of redox systems due to compartmentalization and macromolecular complexes identifies a need to understand redox communication between these compartments/complexes. Because there are many thiol/disulfide elements in proteins and their interaction rates are highly variable, effort is needed to develop a standardized means to define redox pathways. This problem may be addressed by functionally mapping protein cysteines according to their interactions with GSH and thioredoxin systems. Finally, there is a need to address the existence of orthogonal redox control elements. Many proteins and functional pathways have multiple redox-sensitive elements. Separate control by GSH and Trx allow different regulatory events to be controlled independently. Hence, different regulatory mechanisms can be considered to be orthogonal. Several examples are now known for such orthogonal regulation, but additional studies are needed to determine the generality of this type of regulation.
Table 1
Evidence that mitochondrial Trx2 is regulated independently of Trx1

<table>
<thead>
<tr>
<th>Evidence</th>
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<tr>
<td>Glc- and Gln-free medium selectively oxidizes cytoplasmic Trx1 and mitochondrial Trx2.</td>
<td>[71]</td>
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<td>Chemical toxicants targeting mitochondrial electron transport show differential effects on Trx1 and Trx2</td>
<td>[222]</td>
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<tr>
<td>Toxic metals oxidize Trx2 much greater than Trx1</td>
<td>[72]</td>
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<td>Trx2 but not Trx1 is oxidized by TNF-α</td>
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<td>tBH preferentially oxidizes Trx2 as compared to Trx1</td>
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### Table 2

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<th>Evidence that nuclear Trx1 is regulated independently of cytoplasmic Trx1</th>
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<tr>
<td>Trx1 accumulates in nuclei following oxidative stress and other stimuli</td>
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<td>Steady-state redox is about 20 mV more reduced than cytoplasmic values</td>
<td>[4]</td>
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<td>High-dose peroxide causes more extensive oxidation of nuclear than cytosolic Trx1</td>
<td>[225]</td>
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<td>The time course of recovery of nuclear Trx1 is slower than cytosolic Trx1</td>
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<td>EGF stimulates oxidation of cytosolic Trx1 but not nuclear Trx1</td>
<td>[5]</td>
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<tr>
<td>Transfection with NLS-Trx1 stimulates transcriptional activation more than Trx1</td>
<td>[133]</td>
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<td>Glc- and Gln-free medium selectively oxidizes cytoplasmic Trx1</td>
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### Table 3
Thiol/disulfide redox controls in human plasma and extracellular compartment

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<th>Association of oxidative stress-mediated pathologic circumstances with plasma thiol/disulfide redox state in human studies</th>
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<tr>
<td>Decreased glutathione availability, oxidation of GSH/GSSG redox in the alcoholic lung</td>
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<td>Antioxidant supplementation decreases CySS concentration in human plasma</td>
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<td>Diurnal variation of Cys/CySS and GSH/GSSG redox states in human plasma; age-dependent diurnal variation in Cys/CySS redox state</td>
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<td>Plasma Cys/CySS and GSH/GSSG redox state in early atherosclerosis</td>
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<td>Age-dependent oxidation of GSH/GSSG and Cys/CySS pools in human plasma</td>
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<td>Plasma antioxidant status after high-dose chemotherapy</td>
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<td>Plasma GSH/GSSG redox state: association with aging, age-related macular degeneration, and diabetes</td>
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<th>Effects of variation in extracellular Cys/CySS redox state on cell functions in in vitro studies</th>
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<td>GSH-independent control of extracellular Cys/CySS redox control in human colon cancer cells, HT-29</td>
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<td>Extracellular Cys/CySS redox controls lung fibroblast proliferation and matrix expression</td>
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<td>Oxidized extracellular Cys/CySS redox promotes monocyte adhesion to vascular endothelial cells</td>
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<td>Oxidation of extracellular Cys/CySS in human retinal pigment epithelial cells (hRPE) sensitizes oxidant-induced apoptosis</td>
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<td>Extracellular Cys/CySS redox-dependent cell growth signaling in colon carcinoma cells is mediated by a metalloproteinase-dependent mechanism involving EGFR</td>
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<td>Cell proliferation in human colonic epithelial cells (Caco2) and hRPE cells were greater at more reduced E_h values</td>
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