Annu. Rev. Pharmacol. Toxicol. 2006. 46:215–34
doi: 10.1146/annurev.pharmtox.46.120604.141122

First published online as a Review in Advance on September 7, 2005

NUCLEAR AND MITOCHONDRIAL COMPARTMENTATION OF OXIDATIVE STRESS AND REDOX SIGNALING

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Key Words glutathione, thioredoxin, transcription factors, green fluorescent protein, redox Western analysis

■ **Abstract** New methods to measure thiol oxidation show that redox compartmentation functions as a mechanism for specificity in redox signaling and oxidative stress. Redox Western analysis and redox-sensitive green fluorescent proteins provide means to quantify thiol/disulfide redox changes in specific subcellular compartments. Analyses using these techniques show that the relative redox states from most reducing to most oxidizing are mitochondria > nuclei > cytoplasm > endoplasmic reticulum > extracellular space. Mitochondrial thiols are an important target of oxidant-induced apoptosis and necrosis and are especially vulnerable to oxidation because of the relatively alkaline pH. Maintenance of a relatively reduced nuclear redox state is critical for transcription factor binding in transcriptional activation in response to oxidative stress. The new methods are applicable to a broad range of experimental systems and their use will provide improved understanding of the pharmacologic and toxicologic actions of drugs and toxicants.

INTRODUCTION

Mediators of oxidative stress, i.e., reactive oxygen species (ROS), also function as second messengers in signal transduction. Effective transmission of information requires specificity, and how ROS signaling occurs with specificity and without oxidative damage remains poorly understood. Specificity in biologic processes normally is determined by characteristics such as binding affinities, reactivities, and spatial distribution of interacting components. For redox signaling, this involves ROS signal generators and sensors as well as the molecular complementarity and structural organization of the target macromolecules containing reversibly oxidizible elements, such as cysteine and methionine residues. Because of the low concentrations of ROS in cells, the high reactivities of the ROS, the analytic difficulties associated with measurement of ROS in cells, and the artifacts produced

by cell disruption, delineation of specificity in redox signaling has been slow despite rapid progress in understanding the relevant macromolecular machinery.

An alternative approach to investigate the specificity of signaling has been focused on oxidation of thiols, presumed targets and mediators of ROS signals. Developments in two areas of thiol oxidation have set the stage for rapid progress in understanding redox compartmentation as a mechanism in specificity of oxidative stress and redox signaling. These include immunochemical methods to quantify redox changes in thiols in specific proteins and fluorescent reporters of thiol/disulfide redox state suitable for study of redox changes in organelles in living cells. In combination with molecular targeting of proteins to organelles, these approaches provide powerful means to elucidate compartmental functions. In this article, we review these approaches and their application to redox compartmentation of oxidative stress and redox signaling in the nucleus and mitochondria and also at the cell membrane. These organelles have distinct functions and differ in many specific properties, including protein composition, pH, concentrations of antioxidants, and sources of ROS generation. Application of the new methods shows specific generation of ROS in the cytoplasmic compartment during EGF-receptor tyrosine kinase activation and compartmental specificity in nucleocytoplasmic redox control of Nrf2-dependent transcriptional activation. Extension of these methods to other pathways can improve the mechanistic understanding of redox signaling and control and help define the transition from normal redox signaling to pathogenic oxidative stress.

New Definition of Oxidative Stress Recognizes a Central Role for Thiol/Disulfide Redox in Physiologic Mechanisms of Signal Transduction

Oxidative stress was defined as an imbalance of prooxidants and antioxidants (1) before the recognition that ROS have central functions in signal transduction. Although this definition retains an appealing simplicity and is relevant to clinical conditions where deficient antioxidants result in disease, several lines of evidence indicate that oxidative stress would be better defined in terms of disruption of redox signaling and control. The most significant argument comes from the standpoint of human intervention studies (2). Even though substantial evidence implicates oxidative mechanisms in major chronic diseases, large-scale trials using antioxidant supplements largely failed to show protection against disease outcome. Disruption of redox signaling and control can occur either with or without a change in overall balance of oxidants and antioxidants. For instance, redox cycling agents and metal ions can selectively disrupt signaling pathways, and addition of antioxidants cannot protect against this disruption. Moreover, some antioxidants become prooxidants at increasing concentrations, a concept that is antithetical to the definition of oxidative stress in terms of a balance between prooxidants and antioxidants. These observations, plus an appreciation of the fundamental importance of redox mechanisms in signal transduction, leads to the interpretation that a redefinition

of oxidative stress as a disruption in redox signaling and control would better direct research toward discovery of key events and therapeutic interventions to improve health outcome for diseases in which oxidative stress is a significant component.

A circuitry model for redox signaling and control has been developed based on the observations that three major thiol/disulfide couples, namely glutathione (GSH)/glutathione disulfide (GSSG), reduced thioredoxin [Trx-$(SH)_2$]/oxidized thioredoxin (Trx-SS), and cysteine (Cys)/cystine (CySS), are not in redox equilibrium and therefore could function as control nodes for many different redox-sensitive processes (3). In this model, redox switches and pathways exist in parallel circuits, with electron flow from NADPH as a central electron donor to ROS and O_2 as electron acceptors. The lack of equilibration of the Trx-1$(SH)_2$/Trx-1(SS), GSH/GSSG, and Cys/CySS redox couples provides evidence that circuits that rapidly interact with one couple do not similarly interact with another couple because this would result in equilibration of the systems. The reducing force of these nodes in different subcellular compartments can be quantitatively expressed in terms of redox potentials for the respective couples, calculated using the Nernst equation; this provides a means to evaluate oxidative stress and redox signaling in these compartments.

NUCLEAR COMPARTMENTATION OF REDOX SIGNALING AND CONTROL

Early Studies on Nuclear Compartmentation of GSH are Contradictory

Multiple studies have illustrated the importance and multifunctionality of GSH in the nucleus. GSH is important in the regulation of the nuclear matrix organization (4), maintenance of cysteine residues on zinc-finger DNA binding motifs in a reduced and functional state (5), chromosome consolidation (6), DNA synthesis (7), DNA protection from oxidative stress (8), and protection of DNA-binding proteins (9).

An exact measurement of GSH in the nucleus is difficult. Common subcellular fractionation techniques of cytosol and nuclei are not useful because GSH is easily lost from nuclei during the isolation process. Most approaches to this problem use thiol-reactive dyes or antibodies to verify GSH localization, but results are contradictory. Bellomo and colleagues (10, 11) used monochlorobimane to label GSH in rat hepatocytes and found that GSH largely resides within the nucleus, approximating a ratio of 3:1 (nuclear: cytoplasmic). However, Briviba et al. (12) showed that microinjection of the conjugate (GSH-bimane) targeted the nucleus, indicating that one could not use the monochlorobimane approach to measure the nuclear pool. Jevtovic-Todorovic & Guenthner (13) showed that a unique nuclear compartment of GSH exists but that this is a relatively small pool resistant to depletion by BSO. More recent studies with HeLa cells and lymphocytes showed nuclear

accumulation of GSH using a GSH-labeling reagent, 5-chloromethylfluorescein diacetate (CMFDA), and confocal microscopy. These studies suggest a role for Bcl-2 in the import of GSH into the nucleus (14). Contradictory to these findings, Cotgreave and coworkers (15) used a polyclonal antibody specifically raised against GSH. Their results showed that in A549 cells, the nuclear and cytoplasmic GSH pools were not in equilibrium, and furthermore, a GSH gradient was also evident in the mitochondria. Although nuclear pools of GSH are doubtlessly important, measurement of nuclear GSH concentrations are limited by current methodologies, and no methods are available to measure nuclear GSSG. Thus, the question of differential cytoplasmic and nuclear GSH pools remains largely unanswered.

New Methods are Available to Measure Nuclear Redox State

A modification of a standard Western blot allows quantification of the redox state of specific proteins by separation of reduced and oxidized forms by gel electrophoresis and detection of both forms with an antibody to an epitope that does not undergo oxidation-reduction (Figure 1). Quantification is obtained directly from the relative intensities of the different bands. Two forms of the redox Western blot have been developed, separating on the basis of differing charge (thiols are modified with a charged alkylating reagent) or mass (thiols are modified with a high-mass alkylating reagent). An alternate technique utilizes antibodies that detect only the oxidized form of a protein (16); extent of oxidation is determined by comparison of the amount of the oxidized form to the total determined by an antibody that does not distinguish redox forms. In both approaches, care must be taken to assure accurate quantification of the oxidized form relative to the reduced form. Additional approaches using mass spectrometry are also being developed (17). The mass spectrometry approaches have the advantage that they provide the capability to define the redox state of specific thiols in specific proteins.

Another approach to establish the nuclear redox state uses engineered, redox-sensitive green fluorescent proteins (roGFP) targeted to the nucleus (18). Such reporters have been used extensively to measure pH and Ca^{2+} ion concentration; critical issues for the use of such reporters are that they rapidly interact with the species they are reporting and that they do not perturb the system by their presence. These issues have not yet been completely resolved for the available roGFPs redox reporters because they are relatively nonresponsive to physiologic oxidative stimulants. However, the introduction of the redox-sensitive GFPs represents a fundamentally important step in this development.

Oxidized and reduced forms of roGFP2 have different excitation wavelengths (reduced at 490 nm and oxidized at 400 nm) for fluorescence. With the assumption that fluorescence characteristics are unaffected by other components in the nucleus, ratiometric redox calibrations of the isolated protein can be used along with measurements in cells to calculate the redox potential. Addition of an exogenous oxidant, aldrithiol, PMA, or xanthine oxidase caused an oxidation of nuclear roGFP2. As mentioned above, however, neither physiological oxidative treatment, which is

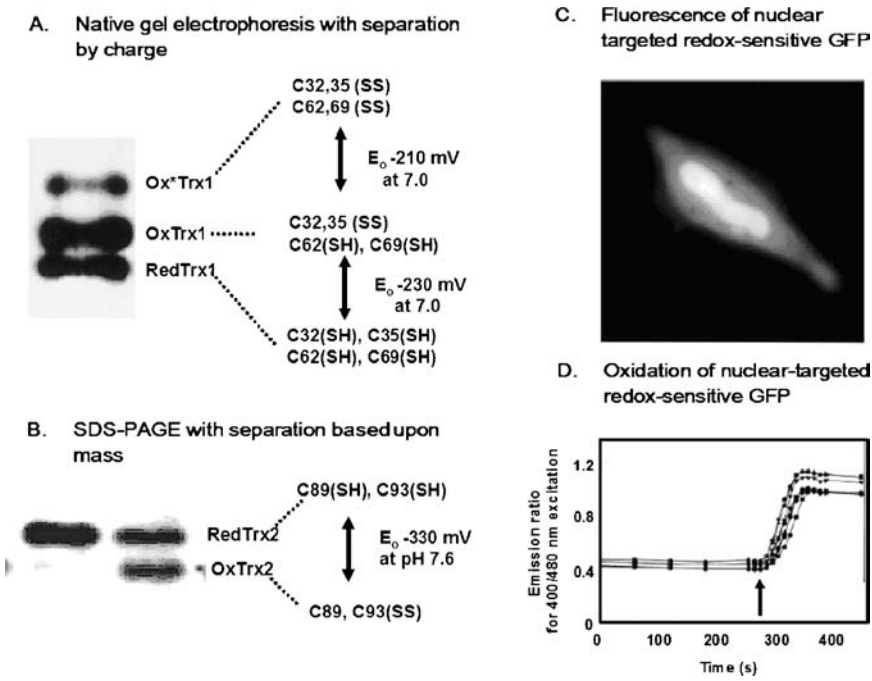

Figure 1 Methods for measuring thiol/disulfide redox in subcellular compartments. (*A*) Redox Western blot allows measurement of thioredoxin-1 (Trx1) redox state in cytoplasm and nucleus. Trx1 contains five cysteine residues, which are normally present as thiols. Two dithiol motifs, one at the active site (C32, C35) and one in a surface α-helix, form intramolecular disulfides during oxidative stress. During redox signaling, only the active site oxidation can be detected. In the analysis, iodoacetic acid reacts with thiols to introduce a negatively charged S-carboxymethyl group; disulfides do not react with iodoacetic acid. The fully reduced form and two oxidized forms can be separated by native gel electrophoresis based on differences in charge and detected by immunoblotting. Redox states in nuclei and cytoplasm are determined by including iodoacetic acid during cell lysis and fractionation. (*B*) Redox analysis of the mitochondrial compartment is performed using a redox Western blot analysis of thioredoxin-2 (Trx2). Trx2 is exclusively found in mitochondria, so an analysis of a cell or tissue extract provides specific information on redox in the mitochondria without fractionation. Analysis is performed following derivatization with AMS. Trx2 contains only two cysteine residues, and the addition of two molecules of AMS increases the mass by approximately 1000 Da. Oxidized and AMS-derivatized forms are separated by nonreducing SDS polyacrylamide gel electrophoresis and detected by immunoblotting. (*C*) Analysis of redox state obtained on a cellular or organellar basis using redox-sensitive GFPs as described by Dooley et al. (18). Fluorescence microscopy of nuclear targeted redox-sensitive GFP (roGFP1) is shown. Alterations in the redox state of the roGFP1 are detected by comparing changes in the emission ratio following excitation at 400 and 480 nm as shown in panel *D*. (*D*) Oxidation is evident with the addition of the exogenous oxidant, aldrithiol to HeLa cells. Panels *C* and *D* are reproduced from Reference 18 with the permission of the publisher.

known to increase ROS level, such as change in pO_2 values ranging from anoxic to hypoxic, depletion of GSH by BSO treatment, nor growth factors including LPA and EGF, caused any detectable change in the excitation ratios of roGFP. Thus, the engineered form roGFP2 shows the feasibility of using a redox reporter to measure thiol-disulfide redox changes in cytoplasm and nucleus, but additional forms to obtain a form with suitable sensitivity to monitor signaling events are needed.

Nuclear Redox State Measured by Trx1

Trx1 is a redox protein found in the cytoplasm and nucleus with many central redox signaling and control functions. Distinct pools are evident, as Trx1 can be imported to the nucleus from the cytoplasm during various forms of oxidative stress (19–21). Mechanisms of specific localization of Trx1 are not known, but the abundance of Trx1 and the presence in both compartments provides the basis for use in the study of nuclear and cytoplasmic redox states.

Redox Western blot methods for Trx redox state are described in detail elsewhere (22, 23). In brief, reduced Trx1 can be labeled with thiol-reactive iodoacetic acid (IAA), adding a negative charge for separation by electrophoresis on a native polyacrylamide gel (Figure 1). Reduced Trx1 that has reacted with IAA runs through the gel more quickly than oxidized, unlabeled Trx1 (24). Following Western blot with an antibody that equivalently detects both forms, densitometric measurements are used for calculation of the redox potential using the Nernst equation (23). This approach was optimized to minimize oxidation during separation of cytoplasmic and nuclear fractions (25).

In untreated THP1 monocytes, nuclear Trx1 redox status was approximately 20 mV more reducing than cytoplasmic Trx1. The addition of an exogenous oxidant, 1 mM *tert*-butylhydroperoxide (tBH), at a concentration that induced apoptosis after 24 h, resulted in oxidation of nuclear Trx1 redox state by 60 mV, whereas cytoplasmic Trx1 was oxidized by only 30 mV (25). Despite these differences in baseline redox state and initial responses to oxidative stress, recovery of both nuclear and cytoplasmic Trx1 was similar during the subsequent 120 min. Thus, nuclear and cytoplasmic Trx1 pools are independently controlled even though they recover similarly after a high-dose oxidative challenge.

Nuclear and Cytoplasmic Redox States Differ During Redox Signaling

Many different processes rely on ROS in signal transduction. 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 (26). An increase in ROS accompanies EGF binding to its receptor, which appears to be a critical component for proper signal transduction as inhibition of ROS effects by the addition of exogenous antioxidants also inhibits the proliferative effects (27). Recent studies on EGF stimulation evaluated ROS production, phosphorylation of the receptor, and oxidation of redox couples

(22). Nuclear Trx1, mitochondrial thioredoxin-2, and cellular GSH did not show any significant oxidation following EGF stimulation. However, cytoplasmic Trx1 was oxidized by nearly 20 mV (22). Although the effects of specific cytoplasmic oxidation of Trx1 are not known, these findings demonstrate that physiologic stimulation can cause the oxidation of specific redox couples in specific subcellular compartments.

Nuclear and Cytoplasmic Redox Couples Perform Distinct Functions During Redox-Sensitive Transcription Factor Regulation

The need for distinct redox regulation in the nucleus and cytoplasm is most apparent in mechanisms whereby cells respond to oxidative stress by transcriptional activation of antioxidant systems. Many transcription factors are redox sensitive, including AP-1, NF-κB, Nrf2, p53, glucocorticoid receptor, and others (28–33). Such sensitivity involves at least two redox-sensitive steps, one in activation of the signaling cascade and another in DNA binding. Additional sites of redox sensitivity for other systems have also been described, including nuclear import (34) and nuclear export (35). Thus, one can expect that many additional redox-sensitive nuclear processes will be described as the newly available methods are more widely used.

Activator Protein-1 (AP-1) was one of the first transcription factors that was studied in the context of redox regulation (Figure 2). Under oxidative conditions, AP-1 activation was shown via the phosphorylation of the Jun protein (36–39). Curran and coworkers first described the redox regulation of AP-1 DNA binding in vitro (40, 41). Treatment of Fos and Jun proteins with NEM in vitro caused an inhibition of DNA binding, but the preincubation with AP-1 oligonucleotide prior to treatment with NEM protected complexes from inactivation. Moreover, treatment with a strong oxidant, diamide, inhibited DNA binding. Together, these findings suggested that cysteine residues specific to the DNA binding domain were the site of redox regulation. Site-directed mutagenesis of cysteine residues in the DNA binding domains confirmed this (28). In this same study, an undetermined nuclear factor was also involved in AP-1 DNA binding. Later, it was discovered that a DNA repair enzyme apurinic/apyrimidinic endonuclease (APE), also termed redox factor-1 (Ref-1), possessed oxidoreductase activity and was responsible for the redox regulation of AP-1 (42). Oxidized AP-1 could be effectively reduced by Ref-1, restoring DNA binding activity. Trx1 was shown to be a critical player in AP-1 regulation as it can reduce oxidized Ref-1 (43). Thus, for AP-1, a nuclear pathway to reduce the Cys of the DNA-binding domain is distinct from the upstream redox events that activate the signaling kinase pathway (Figure 2).

NF-κB is a transcription factor found in many cell types that also has distinct redox-sensitive activation and redox-sensitive DNA binding (44) (Figure 2). In brief, inactive NF-κB resides in the cytosol bound to I-κB. Activation is initiated by I-κB kinase, which phosphorylates I-κB, causing the dissociation with NF-κB and degradation by the proteosome. NF-κB is then free to translocate to the nucleus

(45). Many different physiologic stimuli that activate NF-κB, such as TNFα, are known to produce ROS upon binding to their receptor (46). Chemicals, such as phorbol esters, which also produce ROS, can activate NF-κB (32). In either case, the supplementation of antioxidants or reducing agents blocks the initiation of the NF-κB signaling cascade, implicating ROS as an important component in the initiation of NF-κB signal transduction.

Another level of redox control in NF-κB signaling is also evident, as initial studies utilized the thiol reactive agents N-ethylmaleimide (NEM) and IAA and showed an inhibition of NF-κB binding (47, 48). Inhibition of DNA binding could be prevented by reducing agents, such as β-mercaptoethanol (48). Within the DNA binding domain of the p50 subunit resides a cysteine residue (Cys^{62}), which is redox sensitive. Site-directed mutagenesis of Cys^{62} showed that this cysteine residue mediated DNA binding (47, 49). Thus, ROS production appears to be necessary to initiate the events leading to the dissociation of the NF-κB/I-κB complex, but excessive ROS production can oxidize Cys^{62} and inhibit DNA binding. Overexpression of Trx1 inhibited NF-κB activity, but overexpression of nuclear-targeted Trx1 enhanced it (20). These findings suggest that Trx1 plays distinct roles within the cytoplasm, regulation of activation and dissociation of I-κB, and within the nucleus, regulation of DNA binding (Figure 2).

NF-E2 related factor 2 (Nrf2) is a transcription factor that has been described as regulating anticancer and phase II enzyme genes through the antioxidant response element (ARE) (50). Transcriptional activation by Nrf2 is normally inhibited by binding of Nrf2 to Keap1 in the cytoplasm (Figure 2). Activation is a result of modifications to cysteine residues on Keap1 that allow for the dissociation of the complex to occur, leaving Nrf2 free to move into the nucleus, bind to the ARE and upregulate gene expression (50). GSH, being the most abundant small biothiol in most cells, acts to prevent oxidation and alkylation of Keap1 residues. Conditions that cause oxidation or loss of GSH potentiate Keap1 modification and activate the transcriptional response. Similar to Fos, Jun, and NF-κB, Nrf2 has a cysteine (Cys^{508}) residue in the DNA-binding domain, which must be in the reduced form to bind (30). Upon oxidation of that residue, Nrf2 cannot bind to DNA properly, essentially blocking its ability to upregulate genes even though it has been activated. Nuclear Trx1 reduces Cys^{508} and restores gene expression.

Studies involving selective modification of GSH by metabolic manipulation and Trx1 expression by transient transfection (51) show that the GSH and Trx1 systems have unique functions in Nrf2 activation in the cytoplasm and in DNA binding in the nucleus. Cytoplasmic activation of Nrf-2 was measured by its nuclear translocation, and nuclear activity of Nrf2 was measured by expression of a luciferase reporter construct containing an ARE4 from glutamate-cysteine ligase. Results showed that *tert*-butylhydroquinone (TBHQ), a transcriptional activator that functions through Nrf2/ARE, promoted Nrf2 nuclear translocation by a type I (thiylation) redox switch, which was regulated by GSH and not by Trx1 (51). In contrast, the ARE reporter was principally controlled by nuclear-targeted Trx1 and not by GSH. Thus, the data show that the GSH and Trx1 systems have unique, compartmented functions in the control of transcriptional regulation by Nrf2/ARE.

Additional studies using targeted expression of redox-active proteins have further established specific roles for compartmented redox couples. For instance, peroxiredoxin-5 (Prx5), a protein found in multiple compartments of the cell, is involved in the detoxification of ROS (43), and overexpression of Prx5 in the nucleus protects against peroxide-induced DNA damage (52). Differences in cytoplasmic and nuclear contents of GSSG reductase, GSH peroxidases, and GSH S-transferases have been reported (53), and these differences also may be important in nuclear signaling and control.

MITOCHONDRIAL COMPARTMENTATION OF REDOX SIGNALING AND CONTROL

Redox States in the Mitochondria Represent a Unique Pool

GSH content in cells can range from 2 to10 mM depending on the cell type (15). Total cellular concentrations represent primarily the cytosolic pool, but in hepatocytes where 15% of cell volume is mitochondria, approximately 15% of the total GSH is contained in the mitochondria (54–58). Mitochondrial GSH concentrations generally are similar to those in the cytoplasm (59). Independence of the mitochondrial and cytosolic pools is evidenced by studies using diethylmaleate (DEM) to deplete GSH. Mitochondrial GSH pools were not affected by DEM treatment, but cytosolic pools were depleted by 40% (54, 60). Alternatively, mitochondrial GSH was preferentially depleted by (*R,S*)-3-hydroxy-4-pentenoate (3-HP) as compared to the cytosolic GSH pool (60). Acetaminophen also preferentially depleted mitochondrial GSH as compared to that in the cytosol (61). Estimation of mitochondrial GSH/GSSG redox potentials based on available GSH and GSSG data for mitochondria assuming a pH of 7.8 indicate that values are in the range of −235 to −280 mV (62, 63). Taken together, these studies show the GSH redox independence of each respective compartment.

Mitochondrial GSH can redox cycle via mitochondrial peroxidases and reductases (64), but the mitochondrion does not contain the enzymes necessary to synthesize GSH and rather relies on de novo synthesis and subsequent transport from the cytosol. Transport is stimulated by energizing mitochondria and inhibited by excessive glutamate or disruption of the protonmotive force with proton ionophores (65). Transport of GSH into mitochondria is mediated by two carriers, the dicarboxylate and 2-oxoglutarate transporters (66–69). These anion exchange systems indirectly use the energy of the mitochondrial membrane potential to drive the uptake of the anionic GSH against the prevailing membrane potential (negative inside).

Mitochondrial GSH plays a role in the pathogenesis of many diseases and chemical toxicities. The most widely studied is the effects of ethanol on mitochondrial GSH. A hallmark of alcoholic liver disease (ALD) is the loss of mitochondrial GSH by up to 60% of normal as demonstrated in isolated hepatocytes from ethanol-fed rats (70, 71). Radiolabeling experiments show that cytosolic transport of GSH into the mitochondria is decreased by approximately 35%, an effect that mostly

accounts for mitochondrial GSH depletion with ethanol exposure (72). Although the consequences of mitochondrial GSH oxidation are not completely known, this may regulate the mitochondrial permeability transition (MPT) (73, 74) and directly activate apoptosis. More generally, loss of GSH hypersensitizes the mitochondria to oxidative damage, eventually contributing to necrosis and apoptosis.

Mitochondria contain a unique form of glutaredoxin, glutaredoxin 2 (Grx2), which functions to protect against oxidative stress and prevent apoptosis (75, 76). Grx2 catalyzes the reversible oxidation and glutathionylation of mitochondrial membrane proteins and appears to have a central role in GSH-dependent redox regulation in mitochondria (77). Overexpression of Grx2 in HeLa cells inhibited cardiolipin loss and apoptosis induced by doxorubicin, indicating that Grx2 facilitates maintenance of mitochondrial redox and protects against oxidative stress.

Mitochondrial Trx can be Measured by Redox Western Blot Methodologies

Mitochondria also contain a unique form of thioredoxin, thioredoxin-2 (Trx2), and a specific thioredoxin reductase-2 (TR2), which can reduce oxidized Trx2 using NADPH as an electron donor. Because Trx2 is specific to the mitochondria and is independently regulated, it becomes an excellent candidate for the determination of redox within the mitochondria. The use of antibodies specific to Trx2 allows measurement of mitochondrial redox state by the redox Western blot method without a need for subcellular fractionation. The redox Western blot technique for Trx2 utilizes a thiol-reactive reagent, 4-acetamido-4′-maleimidylstilbene-2,2′- disulfonic acid (AMS), to derivitize reduced Trx2 (22, 78). Samples are separated via nonreducing SDS-PAGE. Derivatized Trx2 with AMS is larger (approximately 1 kDa) and migrates through the gel more slowly as compared to oxidized/underivitized Trx2. Utilizing this method, findings show that *tert*-butylhydroperoxide preferentially oxidizes Trx2 as compared to Trx1 and that Trx2 is resistant to EGF-induced ROS production (22).

Thiol-modified affinity separation followed by a Western blotting has been used to measure oxidation of thioredoxin reductase 2 (TR2) in HeLa cells in response to tumor necrosis factor α (TNFα) (17). In this approach, cell extracts are treated with biotin-conjugated iodoacetamide, immunoprecipitated with an antibody to TR1 (cytoplasmic TR1) or TR2, and probed by Western blotting with HRP-conjugated streptavidin. The results showed a more rapid oxidation of TR2 in response to TNFα with changes apparent at 10 min. Thus, the results show that the mitochondrial TR2/Trx2 is more sensitive to oxidative changes than TR1/Trx1 and that oxidation occurs rapidly enough to serve as an upstream signaling event.

Components of the Mitochondrial Thioredoxin System Protect Against Oxidative Stress

Trx1 demonstrates multiple functions, including ROS detoxification, oxidoreductase activities, and cytokine effects (79). Trx2 functions have not been well

described, but its primary function appears to be the detoxification of ROS through peroxiredoxins. Trx2 overexpressing cells are more resistant to *tert*-butylhydroperoxide-induced cell death (80). Peroxiredoxin-3 (Prx3), the peroxiredoxin found exclusively in the mitochondria, was overexpressed in numerous cancer cell lines and protected against apoptosis (81). Curiously, Patenaude et al. (82) were unable to find any protection by overexpression of TR2 or Trx2 in neuro2A, COS-7, or HeLa cells. These contradictory results may have occurred because there are multiple antioxidant systems in mitochondria. Reduced Trx2 has also been shown to bind to apoptosis-signaling kinase-1 (ASK1), inhibiting apoptosis. Oxidation of Trx2 frees ASK1, allowing apoptosis to ensue (83).

Redox-Sensitive GFP Targeted to the Mitochondria Provides a Useful Tool for Determination of Mitochondrial Redox

As described above for the nucleus, redox-sensitive GFPs also provide a useful approach for determining mitochondrial redox states. Multiple variants of roGFP have been produced, but the proteins that proved most useful were those that showed different fluorescence while in a reduced state as compared with an oxidized state allowing ratiometric methods for quantification (18, 84). One particular form (roGFP1) showed that the reduced form has an excitation at 400 nm for the reduced form, whereas, the oxidized form is excited at 475 nm. The emission wavelengths are the same, but switching between excitation wavelengths allows measurement of redox states. For this, the intensity ratios between the two wavelength pairs are placed in the Nernst equation and used with the midpoint potential to calculate the redox. These constructs can cover a relatively wide range of redox potentials, from −240 to −380 mV; roGFP1 was estimated to have a midpoint potential of approximately −280 to −291 mV (84). Homeostatic potentials in the mitochondria were determined using relative fluorescence ratios and showed that the mitochondria have a reducing redox potential of approximately −360 mV. Because the kinetics of interaction of the roGFPs may be limiting, it is not clear which components are being reported by this redox indicator. However, the method is remarkable in that redox effects can be measured in single mitochondria. Thus, further application of this approach is certain to enhance the understanding of mitochondrial oxidative stress and redox control.

OXIDATIVE STRESS AND REDOX SIGNALING AT THE PLASMA MEMBRANE

A Large Difference in Thiol/Disulfide Redox Potentials for GSH/GSSG and Cys/CySS Exists Across the Plasma Membrane

Extracellular redox signaling and control were recently reviewed (2, 85). The principal features include (*a*) mean plasma GSH/GSSG redox is approximately −140 mV in young healthy individuals. This value is approximately 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. (*b*) Mean plasma Cys/CySS 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. (*c*) Plasma GSH/GSSG and Cys/CySS redox states each are oxidized in association with aging, 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 (86). (*d*) Plasma GSH/GSSG and/or Cys/CySS redox states are oxidized in association with oxidative stress [e.g., smoking (87) and anticancer therapy (88)] and in association with disease [e.g., diabetes (89) and cardiovascular disease (90)]. (*e*) Cells cultured in vitro regulate Cys/CySS redox state to a value similar to that found in vivo in plasma (−80 mV) (91, 92). As described in the next few paragraphs, recent advances have been made in understanding the mechanisms and functional consequences of redox changes in the extracellular compartment.

Oxidized Extracellular Cys/CySS Redox Promotes Monocyte Adhesion to Vascular Endothelial Cells

GSH/GSSG and Cys/CySS are oxidized in association with oxidative stress and in association with cardiovascular disease (90, 93, 94). However, in vitro studies show that cells regulate extracellular thiol/disulfide redox to −80 mV, a value similar to that for the Cys/CySS redox state in plasma. Hwang & Sinskey (95) found that proliferation of cells in culture does not become maximal until the redox state of the culture medium is adjusted to approximately −60 mV. Consequently, even though the extracellular GSH/GSSG redox state appears to better reflect tissue antioxidant defenses (96), the extracellular Cys/CySS redox state appeared more likely to regulate cell functions (85).

Studies of the function of extracellular Cys/CySS show that oxidation of Cys/CySS can potentially contribute in a causal way to atherosclerosis development (92). Effects of extracellular Cys/CySS on early events of atherosclerosis, specifically monocyte adhesion to endothelium, were studied in a model using cultured aortic endothelial cells as a vascular model system. Endothelial cells were exposed to initial Cys/CySS redox potentials ranging from −150 mV (most reduced) to 0 mV (most oxidized). Measurement of molecular processes associated with cell adhesion showed that in comparison to more reduced redox potential, oxidized values stimulated H_2O_2 production but had no effect on nitric oxide (NO) production. The oxidized Cys/CySS potential also activated NF-κB and increased expression of adhesion molecules (ICAM-1, PECAM-1, and P-selectin). Increased expression of the cell adhesion molecules stimulated monocyte binding to the endothelial cells (92). Measurements of cellular GSH showed that this system was not altered by changes in extracellular Cys/CySS redox state under these conditions, and pretreatment with nonpermeant alkylating agents showed that the redox effect was

mediated through thiols that were accessible to the extracellular space. Changes in redox state of plasma membrane proteins were directly detected. Consequently, the results show that the redox state of the Cys/CySS couple in the extracellular compartment can play a key role in regulating early events of atherosclerosis.

Oxidized Extracellular Cys/CySS Redox Sensitizes Cells to Oxidant-Induced Apoptosis

Oxidative stress contributes to the progression of age-related macular degeneration (97) and in vitro studies show that oxidative stress induces apoptosis in retinal pigment epithelial (RPE) cells (98, 99), the cells lost first in development of age-related macular degeneration. Normal human RPE cells in culture were more sensitive to oxidant-induced apoptosis induced by tert-butylhydroperoxide (tBH) under the more oxidized extracellular conditions ($E_h > -55$ mV) compared to the reduced conditions ($E_h < -89$ mV). Mechanistic studies showed that apoptosis was mediated by the mitochondrial pathway because loss of mitochondrial membrane potential ($\Delta\psi_m$), release of cytochrome *c,* and activation of caspase 3 following tBH treatments all increased under the more oxidized conditions. In contrast, extracellular redox state did not affect expression of key ligand-mediated apoptosis machinery, including Fas and FasL. Thus, variation of extracellular Cys/CySS redox state over the range found in human plasma can contribute to a decline in cell populations by enhancing sensitivity to oxidant-induced apoptosis (100). Enhanced sensitivity to apoptosis could provide a general mechanism whereby a more oxidized redox state could contribute to degenerative changes and diseases that are associated with aging.

Extracellular Cys/CySS Redox-Dependent Cell Growth Signaling in Colon Carcinoma Cells is Mediated by a Metalloproteinase-Dependent Mechanism Involving EGFR

Earlier studies show that cell proliferation is dependent on extracellular Cys/CySS redox state and that this mechanism is dependent on growth factor signaling and occurs with no apparent effect on cellular GSH (91). In the absence of added growth factors where a reduced extracellular redox state induced cell proliferation, an 80% increase in EGFR phosphorylation was observed. EGFR phosphorylation was followed by a marked increase in phosphorylation of p44/42 MAPK, and inhibitors of EGFR (AG1478) and p44/42 MAPK (U0126) blocked the redox-dependent p44/42 phosphorylation. Thus, the results show that extracellular redox-induced signaling occurred through EGFR. Pretreatment with a nonpermeant alkylating agent AMS blocked these effects, showing that signaling involved thiols accessible to the extracellular space. Extracellular redox-dependent phosphorylation of EGFR was completely prevented by a metalloproteinase inhibitor (GM6001). An EGFR ligand, transforming growth factor-α (TGFα), was increased in culture medium at more reduced redox states, and an antibody to TGFα partially inhibited the

phosphorylation of p44/42 MAPK by extracellular redox. Thus, the data show that a redox-dependent activation of metalloproteinase can stimulate the mitogenic p44/42 MAPK pathway by a TGFα-dependent mechanism (101). Although the function of this mechanism has not been established in vivo, the results show that physiologically relevant changes in extracellular Cys/CySS redox can affect cell proliferation through a known growth factor–signaling pathway.

MICROCOMPARTMENTATION OF OXIDATIVE STRESS AND REDOX SIGNALING: THE NEXT FRONTIER

Localized Redox Signals Provide a Mechanism to Enhance Recruitment-Activation in Signal Transduction

Earlier studies showed that significant standing diffusion gradients occur under conditions where (*a*) there is a separation of source and sink for a metabolite or ion, and (*b*) the ratio of the metabolic or transport flux relative to the mean concentration is high (102). A general mathematical description for these conditions reduces to the turnover rate for the compartment, wherein empirical data show that turnover rates in biologic systems that are $>2\ s^{-1}$ show evidence of solute heterogeneity (102). For example, mitochondria consume O_2 at a rate of $4 \times 10^{-4}\ Ms^{-1}$; if a multiprotein complex contained NADPH oxidase that converted O_2 to H_2O_2 at 1% of that rate, the rate would be $4 \times 10^{-6}\ Ms^{-1}$. Assuming H_2O_2 is present at a concentration of 10^{-8} M, the site-specific turnover would be $400\ s^{-1}$ and represent a condition where the peroxide could provide a localized oxidation of protein thiols. Thiol oxidation causes structural changes in proteins, which are mostly due to internal disulfide formation, disulfide bridges between proteins, and changes in surface properties owing to cysteinylation or glutathionylation. Such changes alter viscosity in mucus and function in sperm recognition and binding (103, 104). A transient burst of peroxide production that caused localized thiol oxidation could similarly function in cells to stabilize signaling complexes and potentiate signaling by kinase-dependent mechanism. Termination of signal generation could occur by GSH or Trx1-dependent reduction of disulfides and dissociation of the activating complex.

SUMMARY AND CONCLUSIONS

Recent advances provide methods to evaluate compartmental redox state for nuclei, mitochondria, and the extracellular compartment (Figure 3). The redox Western blot analysis allows quantification of organelle-specific redox changes in cell and tissue extracts, whereas use of organelle-targeted redox-sensitive GFPs allows measurement of redox changes in organelles of single cells. The mitochondria have the most negative (reducing) values because of the highly alkaline condition. Nuclei are relatively more reducing than cytoplasm, and cytoplasm is considerably more

reducing than the extracellular space. The endoplasmic reticulum is only slightly more reducing than the extracellular space (105). Functional studies using the redox techniques demonstrate that high-dose additions of oxidants cause global oxidation, but low physiologic levels of oxidant generation result in localized, compartment-specific oxidation. Selective manipulation of GSH and Trx-1 systems further shows that compartment-specific changes are important in redox-signaling and control. These approaches are applicable to a broad range of pharmacologic and toxicologic problems, and their use will allow improved understanding of the mechanistic actions of drugs and toxicants.

ACKNOWLEDGMENTS

The research on which this article was based was supported by NIH grant R01 ES011195, R01 ES09047, and M01 RR00039.

The *Annual Review of Pharmacology and Toxicology* is online at http://pharmtox.annualreviews.org

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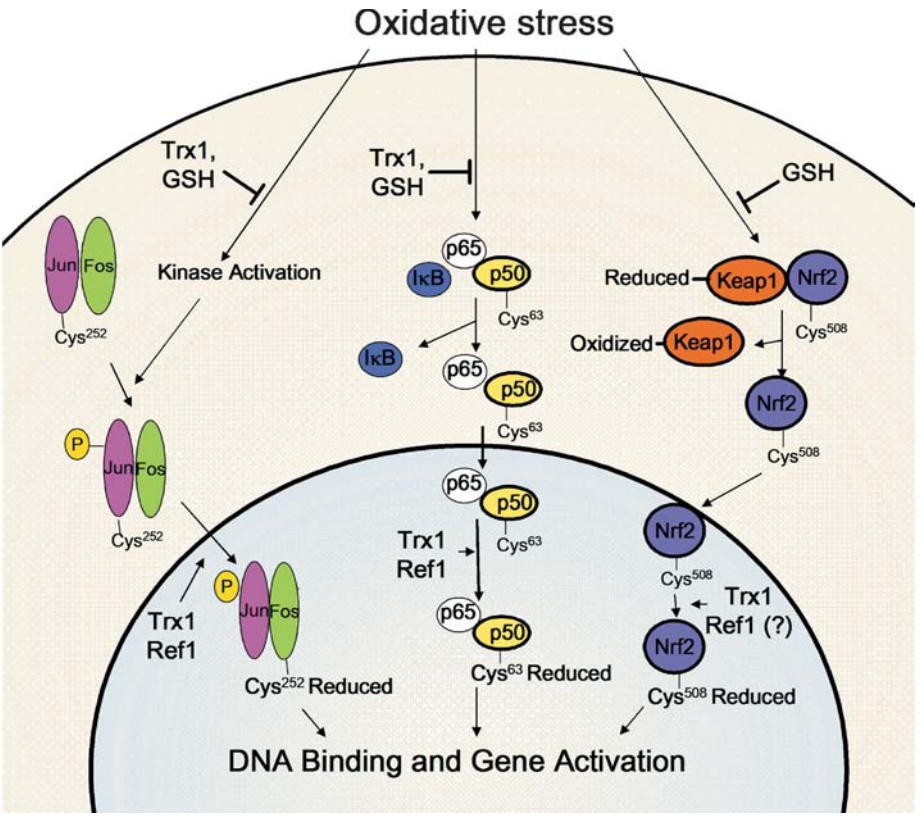

Figure 2 Nuclear and cytoplasmic compartmentation of redox processes in regulation of transcription factors. AP-1, NF-κB and Nrf2 require an oxidative signal in the cytoplasm to initiate signaling for activation (phosphorylation of Jun or dissociation of NF-κB or Nrf2 from inhibitory protein complexes). After activation and translocation into the nucleus, cysteine residues within the DNA binding domain of each transcription factor are reduced by Trx1 and redox factor-1 (Ref-1). Reduction is a prerequisite for transcription factor binding to DNA and subsequent gene activation. Thus, even though oxidative stress in the cytoplasm signals activation, oxidative stress in the nuclear compartment blocks the process.

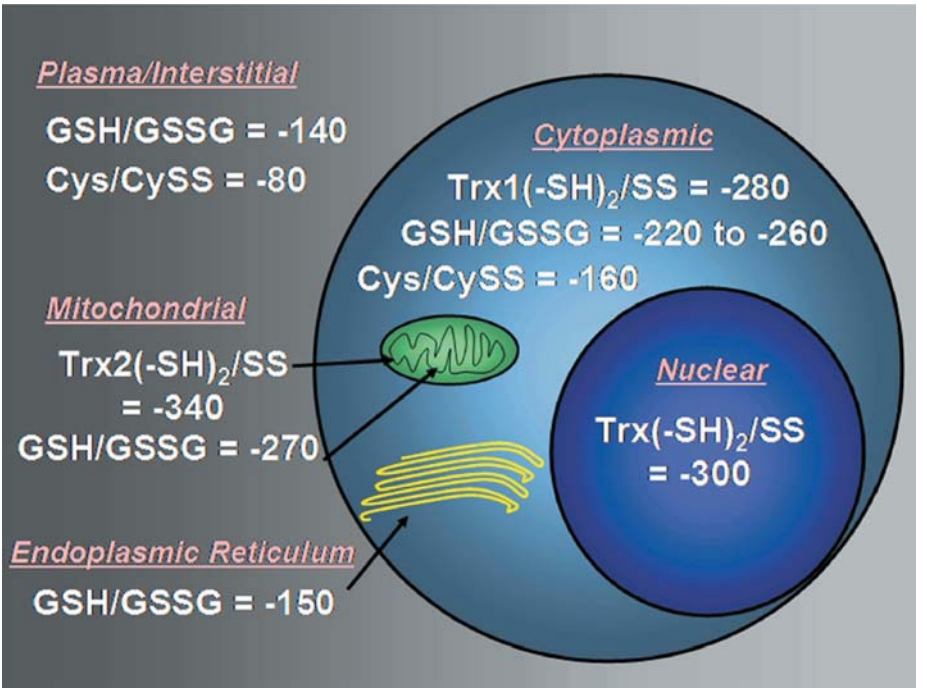

Figure 3 Summary of steady-state redox potentials of GSH/GSSG, $Trx(SH)_2$/Trx(SS), and cysteine/cystine in cytoplasm, mitochondria, nucleus, extracellular space, and endoplasmic reticulum. The presence of these couples in each distinct compartment allows for the independent, specific control of redox processes that occur in these regions.

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Volume 46, 2006

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