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Thiol Oxidation of Cell Signaling Proteins: Controlling an Apoptotic Equilibrium

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Abstract Studies of cell signal transduction have predominantly focused on regulation of protein function by phosphorylation. However, recent efforts have begun to uncover another layer of regulation mediated by direct oxidation of cysteine residues in signaling proteins. Typically induced during signaling responses accompanied by generation of reactive oxygen species, these thiol modifications have a variety of functional consequences for target proteins. Using specific signaling protein targets as examples, we discuss how thiol oxidation generally activates pro-apoptotic signaling pathways while inhibiting pathways that promote cell survival. We propose a model in which thiol oxidation acts to control the equilibrium between survival and apoptosis, fine tuning cellular responses that play a central role in the apoptotic decision-making process. We identify areas of focus for future work, including a better understanding of specificity in thiol oxidation events, and a critical need for approaches to examine these modifications under physiologically relevant signaling conditions. J. Cell. Biochem. 93: 104–111, 2004.

Key words: apoptosis; reactive oxygen species; glutathionylation; sulfenic acid; disulfide; kinase; p53; NF-kappaB

Reactive oxygen species (ROS) have long been known to induce toxic cell responses including activation of stress signaling pathways and apoptosis. However, the means by which redox events regulate these pathways remained elusive. Only recently has a mechanistic understanding of these events begun to emerge, with the demonstration of direct and functionally relevant modification of several critical signaling proteins by oxidation of protein sulfhydryls. Similar to the regulation of signaling molecules by addition of charged phosphate groups, oxidation of protein thiols has structural, conformational, and direct catalytic consequences. Focusing on protein kinases and other signaling molecules with a clear connection to cell survival and apoptosis, we will discuss how protein thiol oxidation may play a critical and perhaps decisive role in fine-tuning signaling responses.

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REACTIVE OXYGEN SPECIES IN SIGNAL TRANSDUCTION

Generation of ROS such as hydroxyl radical, superoxide, and hydrogen peroxide, or reactive nitrogen species such as nitric oxide accompanies diverse environmental stimuli. Importantly, the presence of these second messengers often correlates with subsequent cell death. Cells defend against damage caused by ROS with enzymes that degrade them (e.g., catalase and superoxide dismutase) and "redox buffers" (e.g., glutathione and thioredoxin) that act as scavengers to offset their effects. However, these defenses have a finite capacity to neutralize ROS, and once that capacity is exceeded, the end result is often apoptosis.

ROS-promoted cell death was long thought to be due to non-specific and widespread oxidative damage, but recent work has suggested that ROS may induce apoptosis through a carefully controlled, active process involving direct thiol modification and regulation of crucial components of the cell signaling machinery. These findings imply that ROS may be an active participant in fine-tuning the response of the cell on the basis of variables such as signal intensity, signal duration, or the overall condition of the cell.

In multi-cellular organisms, activation of the apoptotic program in response to oxidative events is viewed as a mechanism to prevent badly damaged cells from later contributing to generation of cancer or other disease processes. We propose a model for ROS-driven thiol modification in which thiol oxidation following a stimulus can shift the balance between survival and apoptotic signaling by activating pro-apoptotic signals while inhibiting survival pathways (Fig. 1). Past a transition point, the cell commits to apoptosis due to this active process.

It is now clear that certain proteins within the cell, and select sulfhydryls within a single protein, are completely oxidized under conditions where other sulfhydryls remain reduced. The mechanism for the specificity of thiol oxidation is unclear. It is possible that thiol oxidation of specific subject proteins results from the intrinsic reactivity of their sulfhydryl residues based on contributions by surrounding residues in the tertiary structure. Alternatively, specific protein sulfhydryls may be targeted for oxidation by as yet unidentified enzymes.

Examining the role of ROS in apoptosis has been technically problematic. Some approaches have limited sensitivity and lack in specificity, for example, the use of fluorescent indicators that quantify ROS. Other approaches rely on indirect evidence based on adding antioxidants (e.g., *N*-acetyl cysteine) to cell culture systems and thus defining "redox-mediated" events. Conversely, hydrogen peroxide or redox-cycling quinone compounds have been used as models to study the effects of intracellular ROS. These approaches have generally supported a role for intracellular oxidative events in promoting apoptosis, but only recently have specific protein targets of oxidation been identified.

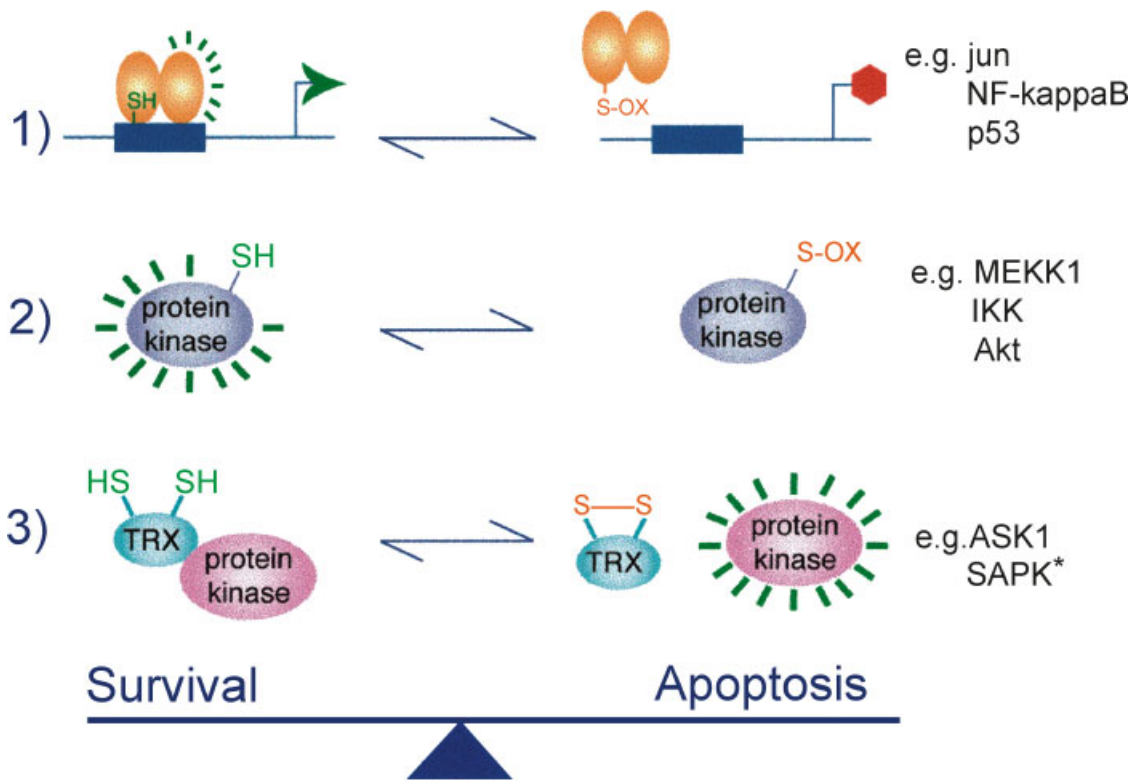

Fig. 1. Model: Thiol Oxidation in the Regulation of Equilibrium between Survival and Apoptosis. Cells exist in a balanced equilibrium between survival and apoptosis. In response to signaling events accompanied by generation of ROS, protein targets are modified on specific cysteine residues, with a variety of functional consequences. These include: (1) inhibition of transcription factors by disruption of DNA binding. (2) Inhibition of protein kinases by direct thiol oxidation and (3) activation of protein kinases by thiol oxidation-dependent dissociation of a bound inhibitory protein (e.g., TRX). In general, thiol oxidation events favor pro-apoptotic signaling (e.g., activation of ASK1) and inhibit pathways that promote cell survival (NF-kappaB dependent gene transcription and Akt). *SAPK is regulated by interaction with GSTpi, and is activated in response to oxidative stress. The role of SAPK in apoptosis remains controversial. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I. Cell Survival and Apoptosis Proteins Regulated by Redox Modifications

	Modification	Outcome	Reference
Survival			
Protein Kinases			
Akt	Intramolecular disulfide	Inhibition	[Murata et al., 2003]
IKK	Undefined oxidation	Inhibition	[Korn et al., 2001]
MEKK1	Glutathionylation	Inhibition	[Cross and Templeton, 2004]
PKC epsilon	Cysteinylation	Inhibition	[Chu et al., 2003]
	Disulfide	Inhibition	[Chu et al., 2001]
	Glutathionylation	Inhibition	[Chu et al., 2001]
SAPK	Indirect (GSTpi)	Inhibition	[Adler et al., 1999]
Transcription factors			
Jun	Glutathionylation	Inhibition	[Klatt et al., 1999]
NF-kB p50	Glutathionylation	Inhibition	[Pineda-Molina et al,. 2001]
	Sulfenic acid	Inhibition	[Pineda-Molina et al., 2001]
p53	Undefined oxidation	Inhibition	[Buzek et al., 2002]
Pro-apoptotic			
Protein Kinases			
ASK1	Indirect oxidation (TRX)	Activation	[Saitoh et al., 1998]
	Indirect oxidation (GRX)	Activation	[Song et al., 2002]
	Indirect S-nitrosyl (TRX)	Activation	[Sumbayev, 2003]
PKC delta	Cysteinylation	Activation	[Chu et al., 2003]

OXIDATION OF SPECIFIC PROTEIN TARGETS

Each of the proteins discussed below, as summarized in Table I, is impacted by direct thiol oxidation of the protein itself or of an interacting regulatory molecule. Cysteine residues can be modified in several ways following oxidative stress events in cells. These include: (1) formation of inter- or intramolecular disulfide bonds, (2) formation of mixed disulfides with a small intracellular thiol (e.g., glutathione, GSH), referred to as glutathionylation, glutathiolation, or S-thiolation, (3) oxidation of the cysteine sulfhydryl to sulfenic acid (R-SOH), or to higher order sulfinic ($R\text{-}SO_2H$) or sulfonic ($R\text{-}SO_3H$) acids, and (4) modification by reactive nitrogen species (i.e., S-nitrosylation). For the purpose of this review, we will focus on the first three, as the role of nitric oxide as a pro-apoptotic or pro-survival signal remains controversial, as reflected in the mixture of pro- and anti-apoptotic targets of S-nitrosylation. These events have been reviewed extensively elsewhere [Brune, 2003].

Differentiating between the alternative types of thiol oxidation is possible using a variety of experimental approaches and chemical probes, ranging from simple (e.g., non-reducing gel electrophoresis) to complex (e.g., observation of modification by mass spectrometry). Identification of a cysteine oxidation on a given target protein is complicated by the observation that the modifications are not always mutually exclusive. For instance, oxidation of cysteine to sulfenic acid is generally considered an unstable modification, and it is often modeled as an intermediate in progression to other, more stable oxidation events. Cysteine residues that are oxidized to sulfenic acid are susceptible to higher order oxidation to sulfinic or sulfonic acid, resulting in irreversible effects on protein activity. Alternatively, sulfenic acid can react with a closely approximate cysteine residue leading to formation of a more stable disulfide bond, in a process likely to protect the protein from irreversible higher order oxidation. However, some examples of stable sulfenic acid oxidation events have also been detected. In other cases, a single target protein (and perhaps even a single cysteine residue) can be susceptible to multiple types of modification in experimental systems. For example, PKC epsilon is glutathionylated, cysteinylated, and forms disulfide bonds under various experimental conditions [Chu et al., 2001, 2003]. Approaches for identifying protein thiol modifications that occur in intact cells under physiologically relevant oxidative conditions will be critical.

SPECIFIC EXAMPLES OF SIGNALING PROTEINS REGULATED BY THIOL OXIDATION

The Apoptosis Signal-Regulating Kinase, ASK1

As the name implies, the apoptosis signal-regulating kinase (ASK1) plays a critical role

in induction of apoptosis. ASK1 is subject to multiple layers of control in which thiol oxidation plays a crucial part. ASK1 is inhibited by interaction with thioredoxin (TRX) [Saitoh et al., 1998]. As mentioned above, TRX is a ROS scavenger but TRX inhibition of ASK1 is due to direct physical binding. When the cell is exposed to hydrogen peroxide or TNF-alpha, two closely adjacent cysteines in TRX form a disulfide bond, causing TRX to dissociate from ASK1 and resulting in activation of the kinase [Saitoh et al., 1998]. Dissociation of the ASK1/TRX complex can also be induced by S-nitrosylation of TRX following exposure of the cells to S-nitrosoglutathione [Sumbayev, 2003], though physiological stimuli that use this pathway remain to be determined.

ASK1 is also subject to regulation by other redox-related events. Similar to its regulation by TRX, ASK1 is inhibited by interaction with glutaredoxin (GRX), a protein structurally related to TRX [Song et al., 2002]. This interaction is interrupted in a redox-sensitive manner during glucose deprivation. Finally, ASK1 is also inhibited by interaction with glutathione S-transferase mu (GSTmu), in a complex that dissociates after heat shock [Dorion et al., 2002]. However, regulation by GSTmu is distinct from regulation by TRX or GRX since it is insensitive to NAC, suggesting that it may not be a redox-regulated event. Taken together, these studies suggest that the regulation of ASK1 in response to redox active stimuli is complex and multifactorial. However, in general, thiol oxidation of TRX or GRX would tend to activate ASK1, suggesting a partial mechanism for oxidant-induced apoptosis.

MEKK1: A Survival Kinase

Work by our group has revealed a role for redox regulation of MEKK1, a protein kinase with a demonstrated survival signaling function. Though the identity of the pro-survival MEKK1 signaling target(s) remains controversial, MEKK1 plays a critical anti-apoptotic role in cells responding to hyperosmotic shock and microtubule poisons [Yujiri et al., 1998] as well as in an intact animal model system of cardiac pressure overload [Sadoshima et al., 2002]. The signaling function of this kinase is regulated in part by caspase-mediated proteolytic cleavage [Cardone et al., 1997], resulting in a shift from anti-apoptotic to pro-apoptotic signaling targets.

We have demonstrated that MEKK1 is inhibited by glutathionylation in cells exposed to hydrogen peroxide or menadione as a model of oxidative stress [Cross and Templeton, 2004]. This inhibition is reversed by exposure of purified MEKK1 protein to DTT in vitro, and requires a single cysteine residue in the ATP binding pocket. While no physiologically relevant stimulus that induces MEKK1 glutathionylation has yet been identified, inhibition of the pro-survival function of MEKK1 could contribute to the progression of the apoptotic response.

The Stress Activated Protein Kinase SAPK/JNK

MEKK1 is an upstream regulator of the Stress Activation Protein Kinase/jun N terminal kinase (SAPK/JNK) pathway. SAPK is itself subject to redox regulation. Analogous to the inhibition of ASK1 by thioredoxin, SAPK is sensitive to redox regulation through an inhibitory interaction with GSTpi [Adler et al., 1999]. Stimulation with hydrogen peroxide or UV irradiation results in oligomerization of GSTpi and dissociation from SAPK, leading to increased SAPK kinase activity [Adler et al., 1999]. Together with the findings for ASK1, these examples demonstrate a role for TRX, GRX, and GSTpi as sensors of the redox environment that, following oxidation, undergo an apparent conformational change and lose their ability to bind to and inhibit their respective kinases.

In unpublished experiments, we have observed that SAPK is inhibited by direct thiol oxidation during exposure to room air. This artifactual oxidation can be prevented by including reducing agents in the incubation buffers and can be reversed by treating the purified protein with DTT in vitro. It is likely that SAPK is also sensitive to oxidation within cells. Importantly, complete inhibition of SAPK catalytic activity is not accompanied by a decrease in phosphorylation of the activating sites. Thus, the use of anti-phosphoSAPK/JNK antibodies to measure SAPK kinase "activity" would mask the impact of thiol regulation on SAPK signaling, since the inhibitory effects of oxidation on activity are not reflected in the kinase phosphorylation state. It may be generally concluded that while anti-phospho site antibodies are a powerful tool for assaying the activation state of a given kinase, using them exclusively may fail to reveal critical layers of

regulation that result from inputs such as thiol modification.

The role of SAPK in apoptosis remains very controversial. Activation of SAPK frequently accompanies apoptosis, but whether activation is protective, causative, or a by-product of apoptotic signaling remains open to debate. The conclusion seems to depend on the cell type, the duration of the SAPK signal, the nature of the stimulus, etc. In addition, many studies of the role of SAPK in apoptosis rely solely on the use of phospho-antibodies as a measure of SAPK activity. Thus these studies would have failed to detect any effect of thiol modification and inhibition of SAPK catalytic activity.

Other Survival Signaling Kinases

Several other kinases with clear connections to survival signaling have been demonstrated as targets of thiol modification and inhibition. The IkappaB kinase complex (IKK), critical in the activation of the pro-survival transcription factor NF-kappaB (see below) is inhibited by hydrogen peroxide due to a direct, DTT-reversible but as yet unidentified oxidation event [Korn et al., 2001]. The crucial survival signaling kinase Akt/PKB, is inhibited by hydrogen peroxide through a mechanism involving formation of an intramolecular disulfide bond between cysteines at positions 297 and 311, leading to increased association of PP2A, dephosphorylation, and subsequent degradation [Murata et al., 2003]. This inhibitory mechanism can be prevented by overexpression of glutaredoxin. Finally, the PKC isoform epsilon, that provides an anti-apoptotic signal, is inhibited by a thiol-redox mechanism involving glutathionylation, cysteinylation, or potentially disulfide bond formation, depending on the experimental approach [Chu et al., 2001, 2003]. Importantly, in the same experiments, the pro-apoptotic PKC isoform delta retains activity or is even modestly stimulated. These experiments again support the notion that thiol oxidation promotes pro-apoptotic signaling.

Transcription Factor Targets of Oxidative Modifications

Several transcription factors that regulate gene expression programs crucial for survival are also subject to redox regulation. These include the p50 subunit of NF-kappaB and the c-Jun protein that is involved in AP-1 dependent gene expression. Both of these transcription factors are glutathionylated at a cysteine residue in the DNA binding domain, resulting in inhibition of DNA binding activity [Klatt et al., 1999; Pineda-Molina et al., 2001]. Oxidation of p50 by sulfenic acid formation at the same cysteine residue has also been detected [Pineda-Molina et al., 2001]. By interfering with the activity of these transcription factors, oxidative modifications would inhibit the expression of pro-survival proteins, resulting in a pro-apoptotic state.

p53

The p53 tumor suppressor protein is subject to oxidative modifications that have been correlated with effects on DNA binding activity, oligomerization, subcellular localization, and stability [Wu et al., 2000; Buzek et al., 2002; Sun et al., 2003]. p53 activation correlates with an increase in ROS [Polyak et al., 1997] that is important for subsequent apoptosis [Johnson et al., 1996], indicating a pivotal role for p53 mediated gene expression in redox-mediated apoptotic decisions. Wild type p53 induces expression of numerous target genes [Polyak et al., 1997], the best characterized being the cell cycle regulator p21Waf1/CIP1. However, a set of thiol redox regulating genes (e.g., quinone oxidoreductase homolog, proline oxidase homolog, microsomal glutathione transferase homolog, galectin-7 etc. [Polyak et al., 1997]) are also induced, many at levels greater than p21Waf1/Cip1. By this measure, control of the redox environment is a more significant role for p53 than cell cycle control.

Ras

The small G protein Ras is glutathionylated following exposure to hydrogen peroxide in vitro or treatment of intact cells with diamide [Mallis et al., 2001]. H-Ras is also S-thiolated during reperfusion in an isolated ischemic heart model [Eaton et al., 2002]. While the role of Ras in apoptosis is not clear, oncogenic Ras induces senescence in cultured cells. Importantly, expression of activated V12Ras is accompanied by generation of ROS that are important for the downstream signaling processes leading to senescence [Lee et al., 1999].

Protein Phosphatases

A variety of protein phosphatases undergo thiol oxidation at their catalytic cysteine residue, thereby inhibiting phosphatase activity

(for review, see Chiarugi and Cirri, 2003). In contrast to most of the examples discussed above, oxidation and inhibition of phosphatases has been shown to occur during physiologically relevant signaling in response to growth factor stimulation. Among the best-characterized targets is PTP1B, whose active site cysteine is susceptible to oxidation to sulfenic acid following exposure to hydrogen peroxide [Denu and Tanner, 1998]. The sulfenic acid can further react to form a sulfenyl-amide bond with an adjacent amino acid [Salmeen et al., 2003; van Montfort et al., 2003]. When exposed to diamide in the presence of glutathione in vitro, PTP1B is glutathionylated at the active site cysteine, likely through a sulfenic acid intermediate [Barrett et al., 1999]. Either the formation of a sulfenyl-amide bond or glutathionylation protect the active site from higher order, irreversible oxidation to sulfinic or sulfonic acid.

Other phosphatases form disulfide bonds between their catalytic cysteine and closely proximate cysteine residues, also through a sulfenic acid intermediate. These include the tumor suppressor lipid phosphatase PTEN [Lee et al., 2002], the cell cycle regulatory phosphatase cdc25c [Savitsky and Finkel, 2002], and the low molecular weight-PTP (LMW-PTP) [Caselli et al., 1998]. Inhibition of phosphatases by oxidative modification promotes growth factor signaling by transiently inhibiting the antagonistic dephosphorylation of activated signaling proteins. How this might contribute to oxidant-induced apoptosis is unclear.

IRREVERSIBILITY: A REDOX THRESHOLD

Almost all of the thiol modifications discussed above are reversible as demonstrated experimentally by exposure of the isolated protein to chemical reducing agents (dithiothreitol, beta-mercaptoethanol) or biological reducing agents (glutathione, thioredoxin) in vitro. The exception is higher order oxidation to sulfinic or sulfonic acid, that is not reversible by these treatments. The long held model for the characterized targets of thiol oxidation suggests that proteins that are susceptible to sulfenic acid oxidation are protected from irreversible over-oxidation by reaction of the sulfenic acid either with glutathione (resulting in glutathionylation) or with a nearby protein cysteine residue (leading to intra- or intermolecular disulfide bond formation). Both of these events would then be reversed by cellular reducing systems when the redox balance returned to normal. Higher order, irreversible oxidation events are predicted to occur only under extreme oxidizing conditions likely to correlate with induction of apoptosis. This suggests that thiol oxidation, in addition to acting as an adjustable balance between survival and apoptosis could reach a threshold past which the commitment to cell death is irreversible.

Complicating this, recent work has suggested that oxidation of the active site of peroxiredoxin to sulfinic acid is in fact reversible [Woo et al., 2003a]. An enzyme that is important for this process has been identified in yeast and aptly named sulfiredoxin [Biteau et al., 2003]. An apparent homolog of this protein exists in mammalian cells, suggesting that even sulfinic acid modification may be reversible through an enzymatic process.

CHALLENGES FOR THE FIELD: TECHNICAL HURDLES AND PHYSIOLOGICAL RELEVANCE

Progress towards the identification of targets and mechanisms of thiol oxidation in controlling apoptosis requires some specific technical advances.

Determinants of Thiol Susceptibility

At present it is unclear what features contribute to the sensitivity of a given cysteine residue to oxidation. The identity of surrounding residues in the primary sequence or the tertiary structure may play a role in making a thiol more or less reactive. Alternatively, regulation of signaling proteins by thiol oxidation raises the possibility that sulhydryl regulating enzymes like GST and GRX may target specific proteins for oxidation in a manner similar to protein kinases targeting phosphorylation of their substrates. Further studies on the regulation of specificity in thiol modification will be critical.

Improved and Specific Labeling Strategies to Identify Highly Reactive Sulfhydryls

Labeling of reduced sulfhydryls is possible through the use of non-specific *N*-ethyl maleimide or iodoacetamide. Methods to identify inducibly glutathionylated proteins (such as using cell permeable forms of biotin labeled GSH [Sullivan et al., 2000]) are promising. Perhaps similar to phosphoepitope-specific antibodies, it may be possible to generate antibody probes that detect oxidized forms of

proteins. In fact, progress has been made with the development of antibodies that specifically recognize the sulfinic or sulfonic acid oxidized form of peroxiredoxin [Woo et al., 2003b]. A similar approach could be applicable to many of the oxidative modifications described above.

Improved Indicators of the Intracellular Redox Environment

Fluorescent indicators such as dihydrorhodamine have been available for a long time, and are useful for quantifying strongly oxidizing ROS. Promising new reagents such as GFP variants that alter their fluorescence characteristics [Hanson et al., 2004] may prove useful in detecting redox changes near the reducing potential of the GSH:GSSG redox pair. Agents that allow visualization of thiol redox changes in specific subcellular locations will be particularly valuable.

Improved Proteomic Instrumentation and Strategies

Analysis and identification of thiol redox targets within a complex mixture of proteins is an important goal. Progress in this regard has been made to identify thiol redox targets in yeast [Rabilloud et al., 2002], though these approaches will have to be improved to be applicable in mammalian systems.

Physiological Relevance

Most of the thiol oxidation events discussed above have been studied using model systems and/or chemicals that generate a strongly oxidizing environment. Demonstration of thiol oxidation in response to physiologically relevant signaling by growth factors, inflammatory cytokines, etc., must be a focus of future research.

Appreciation of the Contribution of Redox

Finally, and perhaps most important, is an understanding among research scientists that redox chemistry is in constant flux in living cells and plays a critical role in many cellular processes. Students should learn how to understand biological redox in a quantifiable way, and should view it as important as phosphorylation or glycosylation in the control of protein function.

CONCLUSION

Cells have many layers of defense against oxidant-mediated damage. The examples cited above indicate that thiol oxidation of specific protein targets is a part of a precisely controlled and regulated signaling response that leads, in many cases, to apoptosis. Understanding these signaling processes may afford us the ability to manipulate them, allowing us to adjust the balance between cell survival and cell death by using redox active therapeutics to shift the equilibrium.

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