Redox-Dependent Transcriptional Regulation

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Abstract—Reactive oxygen species contribute to the pathogenesis of a number of disparate disorders including tissue inflammation, heart failure, hypertension, and atherosclerosis. In response to oxidative stress, cells activate expression of a number of genes, including those required for the detoxification of reactive molecules as well as for the repair and maintenance of cellular homeostasis. In many cases, these induced genes are regulated by transcription factors whose structure, subcellular localization, or affinity for DNA is directly or indirectly regulated by the level of oxidative stress. This review summarizes the recent progress on how cellular redox status can regulate transcription-factor activity and the implications of this regulation for cardiovascular disease. (Circ Res. 2005;97:967-974.)

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Oxidative stress represents a common threat and danger for all aerobic organisms. Any enzyme capable of metabolizing oxygen also carries with it the intentional or accidental capability of generating reactive oxygen species (ROS). Once formed, these radical species can damage a number of essential cellular components including lipid membranes, DNA, and proteins. In addition, there exists a growing body of evidence that acting at lower concentrations, ROS can be purposely made within cells to serve as signaling molecules.¹ The response of a cell or organism to an increase in superoxide, hydrogen peroxide, or related ROS often involves the activation of numerous intracellular signaling pathways. These cytosolic pathways can, in turn, regulate the activation of transcriptional changes that allow the cell to respond appropriately to the perceived oxidative stress. In addition to the regulation achieved by classical cytosolic signaling pathways, such as the family of mitogen-activated protein kinases (eg, mitogen-activated protein kinase, c-Jun N-terminal kinase, and p38 kinase family), evidence suggests that certain transcription factors can directly or indirectly alter their activity, depending on cellular redox conditions.

Numerous studies have implicated oxidative or nitrosative stress in the progression of atherosclerosis and heart failure as well as the regulation of angiogenesis and other cardiovascular conditions.² ⁵ Although the evidence for an oxidative stress component to disease initiation or progression has been well documented, the precise and relevant molecular targets of ROS remain less well understood. One clear mechanism through which ROS might alter the vessel wall or the myocardium would be through a redox-dependent change in transcriptional outputs. Clearly, this is not the only mechanism one could envision because the ROS-mediated oxidation of low-density lipoprotein cholesterol represents a transcription-independent mechanism through which oxidants could obviously contribute to atherosclerosis. Nonetheless, understanding how changes in intracellular oxidants might affect transcriptional activity represents an important avenue in understanding how ROS contribute to numerous disease states.

In this review, we have decided to present an overview of a select handful of well-characterized redox-dependent transcriptional regulatory mechanisms. Rather than confine the
discussion to those mechanisms already known to operate in the myocardium or vasculature, we have, instead, purposely broadened the discussion to include a number of model systems ranging all the way from bacteria to mammals. Although some of these examples are known to be directly applicable to cardiovascular disease, others undoubtedly will only be relevant with regard to the more general principles they reveal. It is our belief, however, that the range of examples provided demonstrate the importance as well as the complexity of turning oxidant signals into transcriptional outputs.

**Lessons From Simple Organisms: SoxR, OxyR, and Yap1**

Bacteria respond to either superoxide anions or hydrogen peroxide with the induction of a discrete set of gene products. Interestingly, the bacterial response to elevated but sublethal concentrations of either superoxide or hydrogen peroxide significantly differ, suggesting that even these presumed evolutionary simple organisms have the ability to sense and distinguish subtly different species of oxidants. Early work identified 2 separate families of redox-dependent transcription factors responsible for this dichotomous behavior. The SoxR and SoxS proteins have been demonstrated to orchestrate the bacterial response to superoxide, whereas the OxyR protein is involved in responding to hydrogen peroxide. In both cases, the transcriptional machinery directly or indirectly senses the relevant oxidant species. For the case of superoxide, the radical species appears to modify the activity of SoxR, which, in turn, induces expression of SoxS. Increased levels of SoxS result in increased expression of more than a dozen other genes including enzymes responsible for DNA repair (eg, nfo, a bacterial endonuclease) as well as genes involved in detoxification (eg, sodA, a manganese-containing superoxide dismutase). The capacity for SoxR to sense superoxide resides in its protein structure and, in particular, its iron-sulfur (2Fe-2S) clusters. These metal-based clusters can undergo either a 1-electron oxidation or reduction, switching the protein complex between a reduced or oxidized state. Although both of these oxidation states can bind DNA, only the oxidized form contains an intramolecular disulfide bond between Cys199 and Cys208 that forms only when Cys199 is oxidized to a sulfenic acid (Cys199–SOH) form. Whereas Stamler and colleagues suggest that this structural modification of OxyR by hydrogen peroxide is the formation of a modified Cys199 to a sulfenic acid (Cys199-SO–H) form, the inactive form contains reduced thiols (see Figure 1). This disulfide-bond based on/off model of OxyR was supported by various structural studies of OxyR under oxidized or reduced conditions.

In addition to the disulfide bond–based on/off switch model of OxyR activation, Stamler and colleagues proposed that the OxyR transcription factor functions as an “on/off” switch, in which the active form contains an intramolecular disulfide bond, and the inactive form contains reduced thiols (see Figure 1). This disulfide-bond based on/off model of OxyR was supported by various structural studies of OxyR under oxidized or reduced conditions.

In contrast to the Fe–S–based superoxide sensing system of SoxR, the sensing of hydrogen peroxide by OxyR revolves around cysteine chemistry. Once activated, the targets of OxyR include katG (a hydrogen peroxidase I), gorZ (a glutathione reductase), and oxyS (a small nontranslated regulatory RNA involved in DNA repair). Two particular cysteine residues (Cys199 and Cys208) appear to play an essential role in the activation of OxyR. A cysteine-to-serine substitution of either Cys199 or Cys208 dramatically reduces the activity of the transcription factor. In addition to these 2 critical cysteines, there are 4 additional cysteine residues in OxyR; however, mutations of these other cysteines do not appear to have dramatic effects on OxyR function. A considerable amount of effort has gone into understanding how OxyR function is redox regulated. Storz and colleagues have suggested an attractive model centered on the formation of a disulfide bond between Cys199 and Cys208 that forms only under oxidative-stress conditions. They proposed that the OxyR transcription factor functions as an “on/off” switch, in which the active form contains an intramolecular disulfide bond, and the inactive form contains reduced thiols (see Figure 1). This disulfide-bond based on/off model of OxyR was supported by various structural studies of OxyR under oxidized or reduced conditions.

In addition to the disulfide bond–based on/off switch model of OxyR activation, Stamler and colleagues proposed that the OxyR activity was regulated primarily by the Cys199 residue through various modifications: by oxidative stress to Cys199–SOH, by nitrosative stress to Cys199–SNO, or by forming a mixed disulfide bond (Cys199–S-S-G) with glutathione. Each modified form of OxyR was proposed to have different structure, DNA-binding affinity, and promoter activity. This model suggested a graded response on OxyR transcriptional outputs, depending on the nature of the oxidative stress. Both models appear to agree that the first structural modification of OxyR by hydrogen peroxide is the formation of a modified Cys199 to a sulfenic acid (Cys199–SOH) form. Whereas Stamler and colleagues suggest that this form can activate transcription, Storz and colleagues argue that the sulfenic Cys199 is a chemical intermediate that quickly reacts with Cys208 to form a stable disulfide bond.

Finally, studies in yeast provide another instructive model for transcriptional regulation by redox-sensitive factors. In
Saccharomyces cerevisiae, Yap1 is functionally homologous to bacterial OxyR. In particular, in the face of hydrogen peroxide exposure, Yap1-deleted strains are unable to induce antioxidant genes necessary to protect the organism from oxidative stress. Yap1 is a member of the basic leucine zipper (bZIP) family of transcription factors and is a distant ortholog of the mammalian c-Jun family. Yap-1 can exist in the nucleus, where it is active, as well as in a cytosolic inactive form (Figure 2). Importation of Yap1 into the nucleus is regulated by a nuclear-localizing sequence found in the amino terminus of Yap1, whereas nuclear export is regulated by a C-terminal nuclear export sequence (NES). Similar to OxyR, hydrogen peroxide can induce disulfide bonds between particular sets of cysteine residues in Yap1. Once formed, these disulfide bonds produce a conformational change in Yap1 that masks the NES. This oxidant-induced masking of the NES sequence results in accumulation of the protein in the nucleus and, hence, a mechanism through which oxidants regulate Yap1 subcellular localization and, by extension, regulate Yap1 activity. Genetic and biochemical evidence suggests that the formation of these Yap1 disulfide bonds requires an additional protein, in this case a glutathione peroxidase–related molecule, Gpx3. Interestingly, an analogous regulation appears to occur in Schizosaccharomyces pombe, where the peroxide-sensitive factor is called Pap1.

Mammalian Redox Control: The Biology of Ref-1

The redox regulation of mammalian transcription retains many of the properties already evident in the examples provided by simpler organisms. In particular, the regulation of protein function through cysteine-based oxidation and reduction is a recurrent theme. The importance of this form of regulation is becoming increasingly evident not only for transcriptional regulation but also for other classes of molecules such as protein phosphatases. One important mammalian redox modulator that has been the focus of numerous studies is the bifunctional enzyme, Redox factor-1 (Ref-1) (also termed Ape1, HAP1, and APEX1). Two distinct enzymatic functions have been ascribed to this molecule. The highly conserved C-terminal region of the protein functions as a DNA repair enzyme. In particular, this part of the molecule is involved in the repair of apyrimidinic/apurinic nucleotides (also known as “AP” sites). Such base-pair modifications occur on an ongoing basis as cells are exposed to daily onslaught of ionizing radiation, UV or ROS. The less-conserved N-terminal region of Ref-1 contains the nuclear localization sequence and the redox regulatory domain, which is characterized by 2 critical cysteines, Cys65 and Cys93. These cysteines in Ref-1 are believed to be important in the redox-dependent modification of transcription factors such as activator protein-1 (AP-1) (Fos and Jun), nuclear factor κB (NF-κB), p53, activating transcription factor/cAMP-response element–binding protein (ATF/CREB), hypoxia-inducible factor (HIF)-1α, and HIF-like factor. In general, the oxidized form of these transcription factors have reduced or absent DNA-binding activity, and Ref-1 appears as an important factor for the specific reduction of these transcription factors. Again, the direct oxidation and reduction of the transcription factors usually involves critical cysteine residues within the DNA-binding domain of the protein. Like the cysteine residues in OxyR, the critical cysteines in both Fos and Jun as well as in other Ref-regulated transcription factors appear to be surrounded by a stretch of basic amino acids. This signature is common among “reactive” cysteines. In this case, reactive implies the ability to...
form a thiolate anion (S⁻) at physiological pH. Presumably, the surrounding basic amino acids facilitate this reactivity and distinguish redox-sensitive cysteine moieties from the majority of nonreactive cysteines found within any given structure of a protein.

The isolation of Ref-1 resulted from a series of experiments primarily by Curran and colleagues. Similar to Yap1, the AP-1 transcription factor belongs to the bZIP family of transcriptional activators. AP-1 results from the heterodimerization of Fos and Jun proteins, and this transcriptional complex is important for the proper induction of a number of genes. These AP-1-regulated genes, in turn, allow the cell to respond appropriately to a host of environmental stimuli including, but not limited to, oxidative stress. Hints that reduction and oxidation of AP-1 was important for its biology came from several early observations. Like many other genes involved in growth regulation, c-Jun has been incorporated into an oncogenic retrovirus. Comparison of the sequence of the viral gene v-Jun, with its cellular counterpart c-Jun, revealed that 1 of the amino acid differences in the viral oncogene involved this conserved cysteine residue found within the DNA-binding domain. Interestingly, when the homologous cysteine residue was purposely mutated in the Fos gene, the mutant protein appeared to bind to DNA with greater affinity. In addition, the redox-dependent binding of Fos was abrogated by this mutation, whereas the ability of the protein to induce transformation of cells was enhanced. These results are consistent with the interpretation that cysteine oxidation reversibly reduces DNA binding and, hence, the overall biological activity of AP-1.

The abovementioned studies led to the recognition that the binding of bZIP family members could be regulated by the oxidation-reduction of critical cysteine residues within the DNA-binding domain (see Figure 3). This conjecture was, however, substantially solidified after the biochemical purification of Ref-1. Subsequent immunodepletion analysis identified the 36-kDa Ref-1 protein as the major redox regulator of AP-1 activity in cells. Examination of the structure of human Ref-1 revealed 2 critical cysteine residues at position 65 (position 64 in mice) and position 93. The initial data suggested that cysteine 65 was the biologically important cysteine required for the ultimate reduction of AP-1. This hypothesis has been challenged by recent studies in which this critical cysteine has been mutated without any apparent effect on AP-1 activity. As such, the exact molecular mechanism underlying the redox activity of Ref-1 remains unclear. Indeed, it is not clear whether Ref-1 acts as a direct or indirect redox sensor. For instance, previous studies have indicated that Ref-1 can also bind to the ubiquitous thiol-containing redox protein thioredoxin. Stimuli that activate AP-1 appear to cause the importation of cystolic thioredoxin into the nucleus, where it can bind to Ref-1 and augment AP-1 activity. In many ways, this 2-step process is similar to the paradigm used by Yap1, where a glutaredoxin molecule acts as the redox transducer for this yeast bZIP transcription factor. Finally, although Ref-1 was initially isolated as an AP-1–specific factor, it is now clear that many transcription factors contain critical single or multiple cysteine residues within their DNA-binding domain. Many of these other transcription factors appear to require Ref-1 for optimal activity. As such, the redox-dependent effects of Ref-1 intersect multiple pathways.

The ramification of Ref-1 biology for vascular tissues is the focus of study for a number of laboratories. For instance, recent observations have suggested that in endothelial cells, Ref-1 can inhibit Rac1-dependent activation of the NADPH oxidase, thereby lowering the production of cytosolic ROS, decreasing NF-κB activation, and protecting cells from apoptosis. These activities appear separate from the role of the protein discussed previously. Ref-1 has also been shown to protect against endothelial cell apoptosis induced by cytokines such as tumor necrosis factor-α. Interestingly, mice that are heterozygously deleted for Ref-1 (Ref-1+/−) are spontaneously hypertensive and exhibit impaired vasodilatation resulting from defective endothelial NO production. In another potentially relevant example, Ref-1 has been shown to bind and activate the HIF-1α–inducible transcriptional complex that regulates the hypoxic induction of vascular endothelial growth factor. Interestingly, this response appears to require hypoxia-induced ROS production. These oxidants were shown, in turn, to modify the 3′ guanine within the HIF-1 DNA–recognition sequence. The subsequent ROS-induced formation of an apyrimidinic/apurinic site within this specific region of DNA was shown to facilitate recruitment of a complex containing both HIF-1 and Ref-1. This study, therefore, provides a potential explanation for how the DNA repair and redox functional domains of Ref-1 might be physiologically integrated. It also blurs the line between ROS acting as random damaging agents and ROS-mediated signaling. Another potentially relevant vascular example of Ref-1 biology involves platelet-derived growth factor (PDGF) signaling in smooth muscle cells. In this context, the PDGF-dependent progression from G1/G0 to S relies on the reduction of AP-1 by Ref-1. These results hint at a potentially...
intriguing connection between dysfunction in Ref-1 signaling and smooth muscle proliferation and, hence, the pathogenesis of atherosclerosis.\textsuperscript{48} Finally, Ref-1 also appears to mediate the transcriptional inhibition of renin production, a critical determinant of blood pressure control.\textsuperscript{39}

**Oxidative and Xenobiotic Stress: The Nrf2–Keap1 System**

In mammalian cells, one aspect of the response to oxidants or xenobiotic stress is the coordinated induction of a host of detoxifying enzymes known collectively as phase I and II enzymes.\textsuperscript{49} Among the gene products that are stimulated are enzymes such as NAD(P)H quinone oxidoreductase, glutathione S-transferase, cysteine–glutamate exchange transporter, and the multidrug resistance–associated protein. This coordinated response presumably allows the cell to help inactivate and expel the offending agent as well as to rapidly control and repair the damage. Analysis of common cis-acting elements in the regulatory regions of these and other coordinately induced genes has identified a motif termed the antioxidant responsive element (ARE). Subsequent studies identified the transcriptional regulator Nrf2 as the factor that binds to this element.\textsuperscript{41} Nrf2 is also a bZIP transcription factor that binds DNA as a heterodimer in conjunction with members of the Maf family.\textsuperscript{42} Mice with targeted deletion in Nrf2 fail to robustly induce phase II enzymes in the face of xenobiotic exposure, demonstrating the in vivo requirement of this transcription factor for the overall biological response.\textsuperscript{43,44}

Similar to previous examples of transcription factors that coordinate the oxidative stress response, it is not clear whether Nrf2 directly senses the stress. Two hybrid experiments using Nrf2 as a bait revealed that the transcription factor could bind the cytosolic protein Keap1.\textsuperscript{45} Curiously, Keap1 is a cysteine-rich protein that can bind to the actin cytoskeleton in addition to binding to Nrf2. Cell culture experiments demonstrated that stresses that activate Nrf2 induce the dissociation of Nrf2 from Keap1. Once released, Nrf2 efficiently translocates to the nucleus. As such, these results would suggest that the activity of Nrf2 is negatively regulated by a Keap1-dependent cytosolic sequestration pathway. Interestingly, whereas Keap1−/− mice die shortly after birth, this lethal phenotype can be reversed by the double knockout of both Keap1−/− and Nrf2−/−.\textsuperscript{46} These results again are consistent with the interpretation of Keap1 as a negative regulator of Nrf2 activity and suggest that either too much or too little Nrf2 activity can result in physiological impairment.

The abovementioned in vitro and in vivo studies suggest that the Keap1–Nrf2 interaction is an important regulatory nodal point in the overall response to oxidative or xenobiotic stress. As previously mentioned, Keap1 is a cysteine-rich protein with nearly 5% of the molecule made up of this single amino acid. Of the nearly 25 cysteine residues present, biochemical analysis has identified 5 that are reactive, namely capable of ionization at physiological pH. Of these 5 cysteines, 2 appear particularly critical for Nrf2 regulation.\textsuperscript{47} An attractive model would be that modification of these cysteine residues by ROS-induced stress results in a conformational change in Keap1, allowing for the dissociation of Nrf2. Although such a mechanism is likely, it is important to stress that other regulatory mechanisms, including phosphorylation and proteasomal degradation, are also important in regulating Nrf2 activity.\textsuperscript{48,49} Finally, Nrf2-dependent regulation is beginning to be explored as an important aspect of the cardiovascular system. For instance, this transcription factor has already been implicated in the endothelial cell transcriptional response to laminar flow,\textsuperscript{50} the expression of macrophage scavenger receptors,\textsuperscript{51} and the induction of heme oxygenase in vascular smooth muscle cells.\textsuperscript{52}

**Regulation by Intracellular Redox Buffers**

Although the previous discussion has centered on the transcriptional response to direct oxidative challenge, there is a growing realization that redox regulation extends beyond this paradigm. Cellular redox status is reflected in the balance between a number of reduced or oxidized molecular pairs including GSH/GSSG, NADPH/NADP, and NADH/NAD. The function of a number of transcription factors, as well as important DNA modifying enzymes, appears sensitive to these redox pairs, implying yet another way that cellular redox status is coupled to transcriptional outputs. Three important examples will help demonstrate this point. The transcriptional repressor C-terminal–binding protein (CtBP) was initially characterized as a cellular binding partner for the E1A adenoviral gene product. The interaction of CtBP with E1A resulted in the inhibition of transcription by E1A, a strong viral oncoprotein. Subsequently, CtBP was shown to mediate the transcriptional repression of a number of other transcription partners.\textsuperscript{53} Curiously, the amino acid structure of CtBP revealed a high degree of homology to metabolic enzymes that bind NAD and NADH.\textsuperscript{54,55} Biochemical analysis has subsequently confirmed that this homology is not accidental, as the ability of CtBP to function as a transcriptional repressor is dependent on nicotinamide adenine dinucleotide binding.\textsuperscript{56,57} There is some disagreement as to whether NAD and NADH differ in their ability to regulate CtBP-repressor function. One report has suggested that NAD and NADH are equivalent,\textsuperscript{57} whereas another has suggested that NADH is several orders of magnitude more effective than NAD in regulating CtBP function.\textsuperscript{56} Although it is difficult to accurately assess the physiological ratio of free NAD or NADH in the nucleus, there is some evidence to suggest that the affinity of CtBP is within the calculated range.\textsuperscript{56} In addition, there is some evidence suggesting that more than simply binding NADH, CtBP may act as a dehydrogenase.\textsuperscript{57} At present, the implications of this enzymatic activity are unknown, as is the cellular substrate for CtBP dehydrogenase activity.

Another example that appears to couple transcriptional activity to cellular redox status comes from the analysis of factors that regulate circadian rhythms. A wide range of organisms regulate the level of certain genes based on the daily light/dark cycle. In mammals, a family of heterodimeric transcription factors is essential for maintaining circadian rhythms. These transcription factors include the Clock gene,
NPAS2, and BMAL1. Although the details of the circadian clock and its intricate transcriptional feedback control is beyond the scope of this review, the observation that the DNA binding of both Clock–BMAL1 and NPAS2–BMAL1 heterodimers is sensitive to the NAD(P)/NAD(P)H ratios suggested a mechanism through which environmental inputs could entrain the circadian clock.58,59 In particular, the suggestion was that neuronal or metabolic activity could alter the ratio of NAD/NADH or NADP/NADPH and, thereby, provide a mechanism for which environmental cues could regulate heterodimer binding and, hence, alter circadian rhythms.

Finally, one additional example is relevant to our discussion. The mammalian enzyme SIRT1 (silent information regulator) is the closest ortholog of the yeast enzyme Sir2. Interest in yeast Sir2 increased following the observation from the laboratory of Guarente that overexpression of Sir2 could significantly extend lifespan in at least 2 model organisms.60 Initial analysis of yeast Sir2 function suggested that the protein is essential for transcriptional silencing, a biochemical property that appears to be maintained by the mammalian ortholog SIRT1.61,62 In yeast, Sir2 appears to globally affect transcription by acting as a histone deacetylase. This enzymatic activity has revealed a strict and unique requirement for NAD, and it is now apparent that mammalian SIRT1 is also an NAD-dependent deacetylase.60 Some groups have challenged whether or not subtle shifts in the NADH/NAD redox ratio represents the predominant physiological regulator of SIRT1 or Sir2 activity.63 Nonetheless, there are certain examples already clearly established in which the ratio of oxidized-to-reduced nicotinamide adenine dinucleotides can regulate the activity of SIRT1.64 These and other studies suggest the potential for an important link between redox balance and chromatin dynamics64 and, by extension, a link between redox status and overall gene expression. Finally, there is emerging evidence that SIRT1 may also play an important role in determining apoptotic thresholds in the myocardium.65

Summary
Redox regulation of transcription is an evolutionary conserved strategy in which alterations in intracellular ROS are converted into discrete and reproducible alterations in gene expression (Figure 4). The most well-established examples have come from analyzing the cellular response to exogenous oxidative challenge following the addition of relatively large amounts of superoxide, hydrogen peroxide, or various electrophiles. The cellular response to these challenges involves the robust induction of numerous gene products that primarily function both to inactivate the threat and to restore homeostasis. Although these examples are instructive, they presumably represent the extremes of biological responses. The moderate but sustained rise in ROS levels seen in diseases such as atherosclerosis or heart failure presumably results in smaller but equally regulated alterations in transcription. Nonetheless, it is our belief that the lessons learned from the examples discussed will aid in the understanding of these more subtle disease-associated changes. In particular, the use of cysteine-based sensing as well as the transcriptional modifying effects of redox buffers such as NAD/NADH represent 2 regulatory mechanisms that may help to understand how ROS can initiate, modify, or sustain a wide range of cardiovascular diseases. Significant progress has been made in the last decade regarding the physiological role of ROS in signaling and transcriptional regulation. Although oxidants have been implicated in a wide array of diseases, the discovery that ROS can covalently modify specific residues within specific target proteins, be they cytosolic enzymes or nuclear transcription factors, challenges the preconceived notion that free radicals contribute to diseases solely as random damaging agents. These observations may have important implications for a number of cardiovascular diseases in which oxidative stress is believed to play a significant role.

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References


