Chapter 4  Redox regulation of physiological processes

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“I have learned in the school of life and experience that moderation is the most valuable virtue a citizen can possess” (From An Enemy of the People by Henrik Ibsen, 1882)

In Henrik Ibsen’s famous play “An Enemy of the People”, the town physician Dr Stockmann realizes that his small Swedish town’s medicinal mineral baths, a source of the town’s considerable wealth and fame, are actually contaminated and unhealthy. The local townspeople, rather than reacting with encouragement and support, attack the good physician/scientist and drive him out of town. The mayor, in an effort to convince the doctor of the folly of his ways, argues that “moderation” is the secret, and that those things viewed initially as harmful, when taken in moderation, are naturally beneficial.

The lessons of Ibsen’s play are of some relevance to the study of oxidant signaling. In many cases the progress of the last decade stands as the mirror image of Dr Stockmann’s travails. Indeed for many years physician/scientists were convinced that reactive species such as \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \) and carbon monoxide were toxic substances that caused numerous diseases and pathological states. That view has been altered by the growing realization that in low doses (i.e. moderation) these potentially toxic moieties actually play a beneficial role in normal signal transduction pathways. The list of such species includes nitric oxide, carbon monoxide, \( \text{H}_2\text{O}_2 \) and even hydrogen sulfide. Here we will review the evidence that these molecules are regulated within cells and that they function in a specific and reversible fashion to transmit downstream signals.

4.1.A Nitric oxide signaling
The discovery of nitric oxide (NO) as a signal transduction molecule provided the intellectual framework for many of the other subsequent discoveries in redox biology. Like many great discoveries, the discovery of NO was, however, partly accidental. Furchgott and colleagues were studying the ability of vascular rings to relax or vasodilate in response to an external stimulus such as acetylcholine. When the individual who normally prepared the vascular rings was absent, a substitute technician was enlisted for the experiments. Fortunately or unfortunately, this substitute appeared unable to get vascular ring preparations that would dilate in response to acetylcholine. Curious as to why this was, Furchgott eventually realized that these new ring preparations lacked the normal thin endothelial cell layer. Presumably the new substitute technician failed to carefully prepare the rings, which in turn led to the loss of the normal endothelial layer. This led Furchgott to realize that the endothelium, rather than being a passive barrier was actually involved in a paracrine signaling process. It was subsequently appreciated by others that NO was the molecule that endothelial cells secrete in response to stimuli such as acetylcholine. These discoveries led to the Nobel Prize being awarded in 1998 to Furchgott, Ignarro and Murad.

The biochemical basis of nitric oxide synthesis was significantly accelerated by the discovery and cloning of the enzyme responsible for NO production. This family of enzymes, nitric oxide synthase (NOS), has a unique structure and is encoded by three separate genes. All three enzymes use the common substrate L-arginine to generate NO in addition to L-citrulline. Historically, the first enzyme cloned was neuronal NOS (also termed NOS 1). The structure of the cloned gene demonstrated predicted binding sites for
multiple potential regulatory cofactors including NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, tetrahydrobiopterin and calmodulin. Similar motifs were found in the other two NOS genes termed endothelial NOS (NOS3) and inducible/calcium independent NOS (NOS2). The regulation of enzymatic activity as well as the actual production of NO differs considerably between the various NOS family members. In particular, when NOS2 is activated in immune regulatory cells such as macrophages, large amounts of NO are produced. Activation of this gene product is predominantly through transcriptional mechanisms. In contrast, both NOS1 and NOS3 tend to produce lower amount of NO and the activity of these enzymes is regulated by a rise in intracellular calcium leading to calmodulin-NOS interaction.

While it is impossible to summarize all the information regarding the regulation of NOS enzymatic activity, we would like to briefly discuss one aspect that has particularly important clinical applications. The NOS3 isoform is expressed in the vascular endothelium and is responsible for vasorelaxation of the underlying vascular smooth muscle cells. This is the enzyme Furchgott and colleagues were studying in their original physiological observations. A variety of animal and human evidence suggests that in patients with atherosclerosis, the biological activity of NO is reduced within the vessel wall. The physiological result of this NO deficiency is a clinical syndrome termed endothelial dysfunction. Characteristics of endothelial dysfunction include an inappropriate vasodilatory response to pharmacological agonists such as acetylcholine or physiological stimuli such as increased sheer stress. The development of endothelial dysfunction is thought to precede the development of visible atherosclerotic plaque and a
variety of evidence suggests that patients who develop endothelial dysfunction are at significantly increased risk for subsequent cardiovascular events. An increase in $\text{O}_2^\cdot$ production is thought to be the mechanism by which endothelial dysfunction develops in the pre-atherosclerotic individual. It is well known that $\text{O}_2^\cdot$ and NO can interact to produce the potentially harmful reactive species peroxynitrite. The increase in $\text{O}_2^\cdot$ therefore reduces NO levels through a direct chemical reaction and in turn produces a state characterized by impaired vasodilation.

Clinically, individuals with atherosclerosis or risk factors for atherosclerosis are often put on agents to lower serum cholesterol. The most commonly used agents are the family of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors often termed ‘statins’. These drugs have revolutionized the care of patients with hypercholesterolemia and have significantly reduced cardiovascular mortality and morbidity. While the long term effects of these agents are dramatic, reduction of serum cholesterol takes time. Interestingly, careful kinetic studies in animals and patients suggest that statins can reverse endothelial dysfunction and appear to do so before there is a measurable drop in serum cholesterol. These and other observations have led to the idea of cholesterol-independent effects of statins. Molecular studies have subsequently delineated a plausible explanation for the observed quick reversal of endothelial dysfunction by these agents. This explanation involves an understanding of the post-translational modification that occurs on a family of important signaling molecules, the family of small GTPases. This family includes proteins such as Ras, Rac, RhoA and numerous other similar proteins that regulate a host of intracellular processes. In
particular, as we will discuss later, members of this family are crucial for ligand-stimulated H$_2$O$_2$ production. While a review of the small GTPases is not our intention, a useful framework is to view these molecules as molecular switches that turn pathways on or off based upon which nucleotide (GDP or GTP) is bound to the GTPase. The small GTPases undergo post-translational modification including the addition of isoprenoid intermediates to their carboxyl-tail. The addition of these lipid moieties allows for the insertion of the small GTPases into a variety of biological membranes (Figure 4.1). The synthesis of isoprenoids, like that of cholesterol, is inhibited by HMG-CoA reductase inhibitors. One effect of this statin inhibition is that the Rho subfamily of small GTPases are less abundant in the plasma membrane because of their reduced post-translational modification. An immediate effect of blocking Rho activity is that the activity of a downstream kinase, Akt, appears to be increased. The precise mechanism for Akt activation in this scenario is incompletely understood. It is well known that Akt can directly phosphorylate NOS3 on a particular serine residue and this phosphorylation leads to increased NO production. Similarly, Rho inhibition also results in increased NOS3 mRNA stability leading to increased NOS3 expression. Therefore statins appear to quickly reverse endothelial dysfunction by modulating Rho activity that in turn, increases NO bioavailability through effects on NOS3 phosphorylation and protein levels.

The biological response to NO is often viewed as being two independent arms with both cGMP-dependent and cGMP-independent modes of action (Figure 4.2). Soluble guanylyl cyclase (sGC) exists in the cytosol and the active enzyme is composed of an $\alpha$ and $\beta$ subunit. This heme containing enzyme is responsible for converting GTP
into cGMP. The ensuing production of cGMP is transduced by a variety of important downstream effectors including cGMP-dependent kinases and cyclic nucleotide-gated channels. Interaction of NO with the heme moiety of sGC leads to activation of the enzyme and a subsequent rise in cGMP levels within target tissues. It is believed that the majority of vasodilation induced by NO is due to the rise in cGMP within the underlying vascular smooth muscle cells.

In addition to its effects on sGC, NO can directly modify a number of target proteins through a process sometimes called S-nitrosylation. This post-translational modification results in the reversible, covalent modification of target proteins when NO interacts with a reactive cysteine thiol to form an S-NO protein. A number of proteins appear capable of forming S-NO intermediaries including caspases, phosphatases, signaling molecules (e.g. small GTPases, Src, Ask1) and channels. Some of the S-nitrosylated proteins have a subsequent increase in their enzymatic activity, while in other cases, the reverse is true. The biological importance of this modification is underscored by the regulatory role that a number of target proteins play. Indeed everything from oxygen delivery by hemoglobin, to the transcriptional regulation of gene expression, to overall muscle contraction appears to be modulated in part by NO–protein intermediates. One cell biology example will hopefully underscore this importance.

Exocytosis depends on the fusion of membrane-bound intracellular vesicles with the plasma membrane. One important component of the exocytosis machinery is the N-ethylmaleimide (NEM) sensitive factor (NSF). The fact that this factor displays NEM sensitivity suggests that reactive thiols may play an important role in the underlying
biology. NSF is an ATPase involved in the regulation of two critical components of the exocytosis machinery termed SNAPs and SNAREs. Interestingly, NO treatment appears to block exocytosis and this intervention is associated with the S-nitrosylation of NSF. It was subsequently shown that exocytosis could be restored to previously NO-treated cells when native but not S-nitrosylated NSF was added back. The blocking of exocytosis from endothelial and immune cells may be one way that NO exerts its powerful anti-inflammatory effect.

Finally, the intersection of NO with other ROS is an important area of research. As previously mentioned, NO and $\text{O}_2^- \cdot \text{O}_2$ can form the reactive peroxynitrite and this species is generally viewed as a harmful byproduct. Another pathway in which NO may interact with other ROS is through the regulation of mitochondrial activity. Since the majority of intracellular $\text{O}_2^- \cdot \text{H}_2\text{O}_2$ derives from mitochondrial activity, regulation of mitochondrial biology by NO represents an important potential overlap between ROS species. There is a variety of evidence that NO can bind to cytochrome c present in mitochondria as the terminal electron acceptor. This binding results in an NO-dependent inhibition of electron transport. Less clear is whether the NO that regulates mitochondrial activity is derived from a mitochondrial-specific isoform of NOS. Evidence supporting a mitochondrial NOS (mtNOS) does exist but is not uniformly accepted. Using knockout animals it would appear that mtNOS shares the most similarity with neuronal NOS. There is also growing evidence that NO is an important regulator of the number of mitochondria within cells, a program called mitochondrial biogenesis. Treatment of cells with NO was demonstrated to increase mitochondrial number through a cGMP-dependent pathway.
Interestingly, in mice that underwent caloric restriction, there was an overall increase in eNOS expression. This rise in eNOS was shown to trigger a series of events including mitochondrial biogenesis that appear essential for the longevity associated benefits of caloric restriction.

4.1.B Carbon monoxide signaling

The production of carbon monoxide occurs in a continuous fashion and predominantly results from the enzymatic degradation of heme by the family of heme oxygenase enzymes discussed previously in Chapter 3.7B. High levels of carbon monoxide are toxic. This toxicity results primarily from tissue hypoxia, since the affinity of carbon monoxide for hemoglobin is more than two orders of magnitude higher than the corresponding affinity of oxygen for hemoglobin. Binding of carbon monoxide to hemoglobin which contains four heme iron centers produces carboxyhemoglobin and the allosteric nature of oxygen dissociation insures that carbon monoxide binding to hemoglobin inhibits both oxygen binding and release. Besides hemoglobin, the heme-containing mitochondrial enzyme cytochrome-c oxidase might also play a role in carbon monoxide poisoning.

While clearly toxic at high doses, there is a growing appreciation that like other small reactive molecules, production of carbon monoxide at lower levels might have a signaling function. Perhaps in a counterintuitive fashion, numerous studies have suggested that this low level of carbon monoxide production might provide protection to a number of cellular and organismal stresses. The connection between stress and carbon monoxide production was first appreciated by analyzing heme oxygenase expression.
Two different heme oxygenase enzymes have been isolated. Both HO-1 and HO-2 stimulate the degradation of heme to produce biliverdin, Fe$^{2+}$, and CO. This reaction also requires the presence of molecular oxygen and NADPH. While HO-2 is constitutively expressed especially in the brain and vascular tissues, HO-1 is generally inducible and only seen after exposure to certain stresses. Indeed the discovery of HO-1 was a result of experiments that sought to identify a common 32 kD protein that was induced after fibroblasts where stressed by either UV radiation, H$_2$O$_2$ or sodium arsenite. This protein turned out to be HO-1 and subsequent analysis has solidified the notion that HO-1 expression protects cells from a diverse array of cellular stresses.

A number of putative protein targets for carbon monoxide have been identified, however, for the sake of brevity; we will discuss only a handful which are the most illustrative. Perhaps the most studied target is sGC. Activation of this heme target induces vasorelaxation and is of course the major basis through which NO regulates vascular tone. On a mole per mole basis, compared to NO, carbon monoxide is a much weaker inducer of sGC. Its physiological role remains incompletely defined although it may be most important under circumstances where there is an absolute or relative NO deficiency. Application of carbon monoxide directly to vessels, as well as increased expression of HO-1 in the vessel wall both result in increased vasorelaxation. Interestingly the application of carbon monoxide to vascular smooth muscle cells results in a cGMP-dependent inhibition of smooth muscle cell growth. These results raise the possibility that carbon monoxide might be in part responsible for maintaining VSMC quiescence.
Deregulation of this pathway may contribute to disease states such as atherosclerosis which involve smooth muscle proliferation.

Another interesting example of HO-2 and carbon monoxide-dependent signal transduction comes from some recent studies on the molecular basis of oxygen sensing. It has been known from elegant physiological studies that the carotid body, a small specialized locus of cells in the neck are the main arterial chemoreceptors that sense changes in arterial oxygen, carbon dioxide and pH. The carotid body when activated, releases a number of transmitters that in turn regulate the central respiratory centers in the brain. In particular, a fall in arterial oxygen saturation triggers the carotid body which in turn stimulates an increase in the rate of breathing. The ability of the carotid body to respond to low oxygen resides within a specialized cell called a glomus cell.

Electrophysiological analysis of isolated glomus cells has demonstrated that hypoxia inhibits cell surface K⁺ channels. Inhibition of these channels results in a surge of Ca⁺⁺ entry and subsequent transmitter release. One of the K⁺ channels responsible for regulating glomus cell membrane potential is itself calcium activated. This channel often referred to as BK_{Ca} and physiological studies have suggested that the channel opening is decreased under hypoxic conditions. Nonetheless, relatively little was known regarding how the oxygen sensitivity of channel opening was regulated. A recent study however has implicated the ubiquitous enzyme HO-2 as a candidate oxygen sensor. As mentioned previously, the enzymatic reaction of HO-1 and HO-2 enzymes with heme requires oxygen. Interestingly, HO-2 appears to form a complex with BK_{Ca} and carbon monoxide donating compounds appear to significantly activate the channel (Figure 4.3). Similarly,
knockdown of HO-2 resulted in reduced channel activity. These results support a model where in the presence of oxygen, HO activity is present and the subsequent generation of carbon monoxide modifies the BKCa channel. In contrast, hypoxia inhibits the activity of the channel by the loss of HO-2 generated carbon monoxide production. This inhibition is therefore a direct result of the oxygen dependence of HO-2. Since the initial description of this pathway, a subsequent report has suggested that the alpha subunit of BKCa contains a heme binding domain suggesting that the effects of carbon monoxide on channel activity may be direct.

Another intriguing aspect of carbon monoxide signaling involves the transcription factor, NPAS2. This protein binds to DNA along with another protein partner, BMAL1. Together, these transcription factors have been implicated in circadian rhythms. For instance, knockout of NPAS2 in mice destroys the day-night cycle of expression of the circadian regulated gene Per2. One conserved motif in NPAS2 is the PAS domain and evidence suggests that in simple organisms this domain senses oxygen and does so through the requisite binding of a prosthetic heme group. The ability to bind heme is also seen in NPAS2 although the transcription factor can bind to DNA in both the apo (heme-free) and holo (heme-loaded) state. Interestingly, low micromolar concentrations of carbon monoxide appear to selectively inhibit DNA binding of the holo-NPAS2 form. In contrast, NO did not appear to have such effects at physiological concentrations of the gas. These results raise the possibility that carbon monoxide might regulate circadian rhythms by directly modulating NPAS2 binding. In this regard it is of interest that elevated HO-2 expression is localized to the same regions of the brain that abundantly
express Per2. In addition, heme synthesis appears to be regulated in a circadian fashion. Finally, the requirement for NADPH in the heme oxygenase reaction might allow a connection between metabolism, carbon monoxide production and the circadian clock.

Finally there is growing interest in the therapeutic application of low dose carbon monoxide. A number of in vitro and in vivo studies have suggested that carbon monoxide may have anti-inflammatory, anti-proliferative and anti-apoptotic effects. Some of these effects may be mediated through cGMP dependent pathways. In addition, there is considerable evidence that carbon monoxide may affect certain arms of the mitogen activated protein kinase (MAPK) family. In particular, activation of p38 MAPK by carbon monoxide appears to be important in numerous contexts. The precise mechanism by which these effects are transduced is not known, nor is it clear whether heme sensing plays a prominent role. Similarly, a number of animal models involving both ischemia-reperfusion and solid organ transplantation have suggested that carbon monoxide might provide significant therapeutic benefit. In addition, HO-1 deficient mice appear to have increased mortality following ischemia-reperfusion induced injury and inhaled carbon monoxide appears to at least partially correct this susceptibility. It remains unclear how these beneficial effects are actually modulated and in particular what the relative contribution is for carbon monoxide’s affects on p38 MAPK versus cGMP-dependent pathways.

### 4.1.C Superoxide and hydrogen peroxide

Long believed to be merely toxic byproducts of aerobic metabolism, both superoxide ($O_2^-$) and $H_2O_2$ have emerged in the last decade as bone fide signaling
molecules. At present because of limitations with *in vivo* detection, it is often difficult to determine whether \( \text{O}_2^\cdot \) and \( \text{H}_2\text{O}_2 \) represent distinct signaling molecules. \( \text{O}_2^\cdot \) is relatively short-lived and can rapidly dismutate to \( \text{H}_2\text{O}_2 \) either spontaneously or with the aid of the enzyme superoxide dismutase. Questions remain therefore as to whether these two ROS have shared or different protein targets as part of their signaling pathways. In this regard it is of some interest to note that in *E. coli*, different sensing and effector pathways exist for \( \text{O}_2^\cdot \) and \( \text{H}_2\text{O}_2 \). In this organism, a rise in \( \text{H}_2\text{O}_2 \) is sensed by the OxyR transcription factor while \( \text{O}_2^\cdot \) is sensed by the SoxRS pathway. The activation of these two pathways leads to the induction of two distinct transcriptional pathways including a non-overlapping set of genes involved in DNA repair and anti-oxidant defenses. The basis for sensing these different ROS lies in the primary structure of the OxyR and SoxS proteins. For the case of OxyR, the ability to sense changes in \( \text{H}_2\text{O}_2 \) is secondary to two reactive cysteine residues within the molecule. Some controversy exists regarding the activation of OxyR, as two slightly different models have been proposed. Both involve the oxidation of one reactive cysteine residue from the thiolate anion state (S-) to a sulfenic form (S-OH). In one model, this oxidation is sufficient to induce the full transcriptional activity of OxyR while in the other model this sulfenic residue is rapidly converted to a disulfide bond by reacting with a neighboring reactive cysteine residue. The ability of cysteine residues to undergo a series of reversible oxidation and reduction steps represents a critical aspect of \( \text{H}_2\text{O}_2 \) signaling mechanisms (Figure 4.4). For the case of the SoxS system, the ability to sense and respond to \( \text{O}_2^\cdot \) comes from the prosthetic Fe-S cluster found in the molecule. Oxidation of the iron moiety provides a metal based \( \text{O}_2^\cdot \)
detection system. As such, it would appear that evolutionary simple organisms such as bacteria have evolved to be capable of detecting either \( \text{O}_2^* \) or \( \text{H}_2\text{O}_2 \) and responding to each of these different ROS with distinct genetic programs. It would seem likely that such discrimination would be maintained in higher organisms although the proof of this is still not conclusive. Nonetheless, the use of Fe-based \( \text{O}_2^* \) detection and cysteine-based \( \text{H}_2\text{O}_2 \) detection appears to be a common theme from bacteria to mammalian cells.

Interest in \( \text{H}_2\text{O}_2 \) as a signaling molecules was sparked by the observation that the addition of peptide growth factors such as PDGF and EGF rapidly (e.g. within seconds) induce a burst of production of this oxidant. It was known for many years that phagocytic cells such as the neutrophil could produce large amounts of \( \text{O}_2^* \) and \( \text{H}_2\text{O}_2 \) after stimulation, but the production of these ROS in non-phagocytic cells was not well studied. Perhaps more intriguing than the actual production, was the observation that inhibiting the rise in \( \text{H}_2\text{O}_2 \) resulted in a blunted response to the peptide growth factors. For instance, both EGF and PDGF stimulate the rapid tyrosine phosphorylation of numerous downstream molecules. Interestingly, blocking the rise in ROS appears to block this burst of tyrosine phosphorylation, while the addition of exogenous \( \text{H}_2\text{O}_2 \) stimulates tyrosine phosphorylation. Numerous questions arose from these initial studies including what was the source of ligand-stimulated ROS production and how did a rise in ROS result in increased downstream phosphorylation.

Subsequent studies have provided some preliminary answers to these questions. For instance it now appears that similar to professional phagocytic cells; non-phagocytic cells also have purposeful ROS generators. In the neutrophil, a multi-subunit NADPH
oxidase has been described that contains multiple membrane-bound as well as cytosolic-
recruited proteins. Activation of the NADPH oxidase in phagocytes also requires a small
GTPase protein called Rac2. Once activated, the neutrophil NADPH oxidase produces
large amounts of \( \text{O}_2^{\cdot} \) that is essential for the host defense functions of this cell type.
Recent evidence suggests that the neutrophil oxidase is part of a family of related
oxidases now termed NOX’s that appear to be widely expressed in a variety of tissues.
Molecular studies are still relatively preliminary at this point but most evidence suggests
that the general mode of regulation of various NOX’s may be similar between non-
phagocytic and phagocytic cells. In particular there appears to be an essential role for Rac
family members in regulating ROS levels in different cell types (Figure 4.5). In contrast,
the molecular makeup of the oxidase may be tissue specific especially with regards to the
major cytochrome, which in the neutrophil is the protein originally termed gp91phox. A
number of gp91phox related proteins have now been isolated that appear to be expressed
outside the neutrophil and appear to produce a regulated burst of \( \text{O}_2^{\cdot} \). Levels of ROS are
thought to be several orders of magnitude lower in these newer oxidases when compared
to the neutrophil complex. This lower amount has been viewed as more compatible with
normal signaling. These novel oxidases also have the potential to contribute to cellular
transformation and may play an important role in tumorgenesis. It is interesting to note
that the production of high or low amounts of \( \text{O}_2^{\cdot} \) by the various NADPH oxidases is
reminiscent of the previous example for the various NOS isoforms. In both cases,
professional immune cells appear to produce large amounts of either NO or \( \text{O}_2^{\cdot} \) as part
of their host defense function. In contrast, in other cell types, another form of the enzyme
system, be it NOS or NOX, is assembled to produce the same species of oxidant but at significantly lower amounts. These lower amounts presumably function in normal signaling pathways in these various non-immune cell types.

Similar to the progress that has been made on understanding the potential sources of ligand-stimulated ROS production, there has been considerable progress made on identifying potential specific cellular protein targets of H$_2$O$_2$. Again, here the characteristic that has emerged from an analysis of these target molecules is the common theme of reactive cysteine modification. One class of molecules that is of particular recent interest is the family of protein tyrosine phosphatases and the related family of dual-specific phosphatases. These families of molecules have at their active site a reactive cysteine residue and have long been viewed as potential targets of oxidants. Subsequent in-depth analysis has confirmed and extended this hypothesis, and it now appears likely that ROS represent an important method of phosphatase regulation.

Careful kinetic analysis suggests for the tyrosine phosphatase PTP1B, oxidation of the catalytic and reactive cysteine first produces the sulfenic intermediate (Cys-S-OH). Interestingly, evidence then suggests that this moiety reacts with a nitrogen atom in a neighboring serine residue to produce a five-membered cyclic sulfenyl amide species. It will be interesting to know whether other phosphatases also go through the unusual intermediate following their oxidation. In addition to PTP1B there are a number of other important phosphatases that appear to undergo reversible oxidation and inactivation including PTEN and Cdc25C. Since the observed level of phosphorylation represents the balance between kinase and phosphatase activity, these results suggests that the basis for
oxidant-induced tyrosine phosphorylation may have less to do with actually stimulating kinase activity and more to do with inhibiting phosphatase action.

Another emerging area related to ROS signaling is the regulation of protein function by a process termed glutathiolation. GSH is present in millimolar concentrations in cells and provides an essential role in maintaining a reducing environment within the cell. It represents an important H₂O₂ scavenging system where two molecule of GSH can interact with H₂O₂ to produce two molecules of water and one molecule of GSSG. The GSSG can then be reduced back to GSH by the action of glutathione reductase with NADPH being consumed in the reaction (see Chapter 3.3A). GSSG can directly react with the thiolate anion of reactive cysteine residues in proteins. Alternatively, GSH can directly react with cysteine sulfenic intermediates (S-OH) to also produce a glutathiolated intermediate. In both cases a glutathiolated protein is produced. A number of proteins appear to undergo such modifications following physiological or pathological stress and new methods to more readily detect such intermediaries have emerged. The list of glutathiolated proteins includes transcription factors, metabolic enzymes and structural proteins. Relatively little is known regarding how the cysteine residue of glutathiolated proteins is reduced back to the original thiolate anion although such reversibility would appear essential if glutathiolation is to emerge as means of signaling rather then merely a protective response to oxidative stress.

Taken together, these results suggest that cells purposely produce O₂⁻ /H₂O₂ to both fight infection in the case of phagocytes and to transmit signals in other cell types. Many questions remain regarding how specificity is achieved with ROS dependent
signaling although the spatial confinement of the oxidant signal may be important. In this regard it was recently noted that following ligand addition, the reactive cysteine of the PTP1B located in the plasma membrane was oxidized while the larger cytoplasmic pool of the phosphatase remained reduced and enzymatically active. Presumably this difference in oxidation status and corresponding activity reflects how near the source of oxidant production a specific PTP1B molecule was.

4.1.D Other novel redox molecules

The discovery that NO, carbon monoxide, O$_2^-$ and H$_2$O$_2$ all can act as intracellular messengers has suggested that other small molecules and gases might also be important for biological signaling. In plants for instance, the gas, ethylene, plays a major role in growth, ripening, development and stress resistance. Similarly, less well characterized gases may also be important in mammals. One such molecule whose physiology remains incompletely understood is the molecule hydrogen sulfide (H$_2$S). Formation of this gas is thought to result from the action of either of two different enzymes, cystathionine β-synthase or cystathionine γ-lyase. Both these enzymes are capable of catalyzing multiple enzymatic reactions including producing H$_2$S. Once produced, H$_2$S is a colorless but certainly not odorless gas that readily diffuses across plasma membranes. Similar to both NO and carbon monoxide, H$_2$S is produced abundantly in the brain. This appears to be secondary to the action of cystathionine β-synthase which is highly expressed especially in the hippocampus and the cerebellum. The neuronal actions of H$_2$S have been reported to include regulation of memory and long term potentiation (LTP). This effect is thought to occur through regulation of
NMDA receptor activity. Interestingly both NO and carbon monoxide also regulate LTP although H2S does not appear to regulate cGMP levels like these two other gases. Again, in an analogous fashion to carbon monoxide and NO, H2S can act as a vasoregulatory molecule. The mechanisms underlying this vasodilatation are unclear, although some have speculated that KATP channels are involved. One fascinating application of H2S has been recently reported in mice. Inhalation of H2S resulted in a slowing down of metabolism by approximately an order of magnitude. This effect was completely reversible suggesting that certain non-hibernating mammals could be induced to undergo a state that was metabolically equivalent to hibernation. The ability of H2S to induce mice to revert to a quasi-hibernating state was presumably related to the ability of H2S to reversibly bind to cytochrome c oxidase. This binding, a property also shared by both carbon monoxide and NO, potently down regulates mitochondrial oxidative phosphorylation. The controlled use of such a strategy in patients with ischemia, trauma, or undergoing prolonged surgery may potently reduce tissue injury in these settings.

The last two decades have witnessed an enormous growth in our understanding regarding the biological activity of reactive nitrogen and oxygen species. Although oxidants have long been implicated in the development and progression of various diseases, as well as in organismal aging, the prevailing prejudice was that ROS contribute to these processes through random and non-specific damage to DNA and/or proteins. The discovery that oxidants act as normal signaling molecules raises doubts about whether our previously held prejudices were correct or complete. The next several decades should
provide more insight into this question and should provide significantly more information
on how small diffusible molecules regulate such complex biological systems.

Selected References


Figure 4.1 Statins regulate NO availability. The production of NO is regulated at both transcriptional and post-translational levels. The commonly used drug family, the HMG CoA-reductase inhibitors (statins), blocks the post-translational addition of lipid groups to the Rho family of small GTPases. Rho GTPases negatively regulate endothelial NOS (eNOS) message stability as well as activity. The latter regulation is by altering the phosphorylation of eNOS protein. Negative regulation of Rho activity by statin treatment leads to more NO bioavailability and a reversal of endothelial dysfunction. Interestingly, another small GTPase, Rac1, appears essential for O$_2^-$ production suggesting a critical role for this family of molecules in overall redox regulation. GAPs are GTPase activating proteins and GEFs are GTP exchange factors.
Figure 4.2 NO-dependent signaling. NO regulates numerous important physiological processes. There are at least two major arms of this regulation. The classical arm is through the activation of cGMP dependent signaling. Alternatively, NO can directly modulate protein function through S-nitrosylation (S-NO).
Figure 4.3 Carbon monoxide is an important regulator of hypoxic sensing of the carotid body. Glomus cells in the carotid body respond to low oxygen levels by altering the opening of specialized channels such as $\text{BK}_{\text{Ca}}$ on their cell surface. Channel activity regulates potassium entry and subsequent neurotransmitter release. The activity of the $\text{BK}_{\text{Ca}}$ channel is sensitive to oxygen concentrations and recent evidence suggests that the oxygen-dependent production of Carbon monoxide by HO-2 may be part of the overall hypoxic sensing system.
Figure 4.4 Oxidation of cysteine residues as the basis for H$_2$O$_2$ signaling. Certain reactive cysteine residues are capable of forming a thiolate anion (S$^-$) at physiological pH. These residues can be further oxidized depending on the strength of ROS. Generally the sulfinic and sulfonic forms are viewed as non-reversible modifications, although there is at least one example of the reversibility of a sulfinic-modified protein.
Figure 4.5 Signaling by H$_2$O$_2$. Activation of receptor tyrosine kinases (e.g. PDGFR or EGFR) leads to the activation of PI-3-kinase and the subsequent stimulation of the activity of guanine exchange factors (GEFs). These GEFs can activate small GTPases such as Rac1, leading to the production of H$_2$O$_2$ by the NOX family of oxidases. One class of targets for H$_2$O$_2$ includes the phosphotyrosine phosphatases (PTPs) and the dual specific phosphatases that contain reactive cysteines in their active sites. Inhibition of these phosphatases leads to increased phosphotyrosine signaling (p-Tyr).
4.2 The Role of Nitric Oxide Synthases in Redox Signaling

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The vast number of proteins containing prosthetic groups, such as iron protoporphyrin IX (heme) or FAD or FMN, that facilitate electron transfer, continue to fascinate investigators due to their ability to function in a variety of redox reactions. As demonstrated in multienzyme systems, such as the mitochondrial respiratory system, a number of proteins containing such prosthetic groups can interact in a carefully orchestrated manner to transfer electrons serially from an electron donor prosthetic group of more negative potential in one protein to an electron acceptor prosthetic group of higher potential in another protein. Enzymes have also been discovered that contain several different prosthetic groups of differing redox potentials enabling them to act as miniature electron transfer systems in a single polypeptide chain.

The nitric oxide synthases (NOS) represent such an electron transport system within a single polypeptide chain. The components of these enzymes, so-named because the isoforms produced by three genes in mammalian systems are expressed in various tissues to produce NO for a specific physiological purpose will be addressed. Inducible or macrophage NOS (iNOS) is found in phagocytic cells in which NO is produced for cytotoxic purposes by combining with $O_2^{•−}$ to produce peroxynitrite. Endothelial NOS (eNOS) is located in endothelial cells in blood vessels where NO serves as a vasodilator. Neuronal NOS was first isolated from rat brain neuronal cells and is involved in neurotransmission. The characterization of these isoforms and the reactions catalyzed
will be discussed in order to understand how each of them is regulated and performs its specific function.

4.2.A Characterization of the nitric oxide synthases

Figure 4.6 shows the linear arrangement of the flavin- and heme-containing domains of all three nitric oxide synthases. It can be seen that these enzymes are divided by a calmodulin-binding site roughly into a C-terminus, containing an NADPH-binding site and both FAD- and FMN-binding domains, and an N-terminus containing the heme- and the tetrahydrobiopterin-binding sites, as well as a zinc tetrathiolate.

The existence of such a bi-domain structure was discovered by using protease digestion techniques that proved that the enzymes could be separated into distinct domains and later by heterologous expression of the separate genes in *E. coli* for the flavin- and heme-binding domains that could transfer electrons and bind ligands, respectively. The ability to isolate, purify and crystallize these separate domains has permitted researchers to determine differences among the three isoforms of NOS.

Nitric oxide synthases were first characterized as flavoproteins and were shown to be 50-60% sequence identical to NADPH-cytochrome P450 reductase, an endoplasmic reticulum flavoprotein, also containing both FAD and FMN, essential for electron transfer from NADPH to the cytochromes P450 in a multi-enzyme system that metabolizes therapeutic drugs, steroids, fatty acids and carcinogens (polycyclic aromatic hydrocarbons). With the ability to express the genes in both mammalian and prokaryotic cells, it became possible to purify sufficient quantities of protein to characterize the NOS isoforms as flavin- and heme-containing proteins that could bind tetrahydrobiopterin in
the heme domain. Spectroscopic techniques determined that these enzymes were heme proteins that exhibited a reduced, CO-bound difference spectrum that absorbed visible light at approximately 450 nm, similar to the cytochromes P450.

With this discovery, investigators began to make comparisons between the mechanisms involved in electron transfer within NADPH-cytochrome P450 reductase and neuronal NOS. Later studies confirmed the mechanistic similarities between the flavoprotein moieties of cytochrome P450 reductase and the NOS isoforms and it has been shown that both enzymes produce stable neutral blue semiquinone forms upon reoxidation of flavin hydroquinone forms by either artificial electron acceptors (such as heme-Fe$^{+3}$) or molecular oxygen (Figure 4.7).

Thus, catalysis by both cytochrome P450 reductase and NOS appears to involve redox cycling between 1-, 2-, and 3-electron reduced states of these flavoprotein moieties in order to permit the reduction of the protoporphyrin-bound Fe$^{+3}$, which is a 1-electron acceptor.

4.2.B Regulation of nitric oxide synthases by intrinsic elements

The real puzzle, yet to be solved, is the conformational changes that must occur in order to accommodate these redox events during catalysis by the NOS isoforms, since they function as dimers. Although no full-length X-ray structures are yet available, structural studies have thus far revealed that the expressed heme domains are dimeric in themselves, featuring over 3000Å of dimer interface and a single Zn tetrathiolate involving identical cysteine residues from each monomer (Figure 4.8A). A recent X-ray structure of the flavoprotein domain of nNOS also shows a dimeric conformation (Figure
4.8B), although solution techniques, such as analytical ultracentrifugation of these constructs, have not yet verified this structure. It is probable that until a full-length structure of one of the NOS isoforms is obtained, the overall relationship of the domains within the holoenzyme dimer will remain unsolved.

The discovery, through sequence alignments of the NOS isoforms with cytochrome P450 reductase and other diflavin (FAD- and FMN-containing) enzymes, indicated that autoregulatory inserts exist within the flavoprotein domains of constitutive isoforms (nNOS and eNOS) of NOS, thus shedding new light on their functional characteristics. It is important to point out that iNOS from phagocytic cells is regulated at the transcriptional level, while the constitutive NOS isoforms are regulated at the post-translational level. Therefore, the existence of additional residues (~50) in nNOS and eNOS within the FMN-binding domain, compared to cytochrome P450 reductase and iNOS, raised the possibility that these flexible loops play a role in the regulation of these enzymes. Originally, it was shown that the addition of Ca\(^{2+}\)/calmodulin, required for the interaction of the flavoprotein domain with the heme domain and subsequent electron transfer, caused significant conformational changes in nNOS and eNOS. Also, peptides derived from the autoregulatory insert of eNOS, when applied to either nNOS or eNOS, produced dramatic, titratable inhibition of the electron transfer activities, including NO formation, and this inhibition was reversed by the addition of Ca\(^{2+}\)/calmodulin.

Furthermore, examination of the three NOS isoforms revealed that the C-termini of all three isoforms were between 21 and 42 residues longer than cytochrome P450 reductase, suggesting a functional role for these residues.
The production of mutants of all three NOS isoforms, which did not contain these C-terminal “tails”, showed a dramatically increased electron transfer to all artificial electron acceptors through the flavoprotein domain in the absence of Ca\(^{2+}/\)calmodulin. Interestingly, the addition of Ca\(^{2+}/\)calmodulin, required for electron transfer from the flavoprotein domain to the heme domain for the production of NO, the rate-limiting step for the overall reaction, reduced this increased activity to the level of wild type enzyme. These results, alone, strongly indicate that the C-termini exert a negative modulatory influence on NOS activity and that, in the case of the constitutive NOS isoforms, the addition of Ca\(^{2+}/\)calmodulin relieves this inhibition. Additional studies indicate that these actions occur in concert with conformational changes involving the autoregulatory insert in the FMN-binding domain, Ca\(^{2+}/\)calmodulin binding to the connecting domain between the flavoprotein and heme domains, and the C-termini in constitutive NOS isoforms. In the case of iNOS, the C-terminus is also involved in negative modulation but this is independent of Ca\(^{2+}/\)calmodulin binding since iNOS is expressed with tightly bound Ca\(^{2+}/\)calmodulin.

These intrinsic mechanisms provide regulatory control of NO production within the physiological milieu as influenced by the influx of Ca\(^{2+}\) into neuronal or endothelial cells in the case of nNOS and eNOS. This influx of Ca\(^{2+}\) also influences other extrinsic interactions, such as phosphorylation, particularly in the case of eNOS, by specific kinases. Since cytokines and interleukins control the induction of iNOS at the transcriptional level, extrinsic factors play a major role in the regulation of this NOS isoform.
4.2.C  Extrinsic regulation of nitric oxide synthases

The most studied NOS isoform with respect to post-translational modification is eNOS. The studies, which showed the involvement of protein kinase B (Akt/PKB) in the regulation of eNOS, revealed residues in the flavoprotein domain that were modified by phosphorylation. The C-terminus is most likely involved in the “masking” of the potential phosphorylation site and Ca$^{2+}$ binding influences both the binding of calmodulin and the interaction of NOS with kinases, which also require Ca$^{2+}$.

4.2.D  Interactions of NO with other proteins and enzymes

The first implication of an interaction of NO, produced by chemical means, with a biological entity was published by Ferid Murad, who was awarded the Nobel Prize in 1998 for this discovery. His two fellow Nobelists, Robert F. Furchgott and Louis J. Ignarro, were recognized for their later identification of NO as the endothelium-derived relaxing factor. These findings were profound in discovering one of the physiological consequences of NO in biological systems through its binding to the heme prosthetic group of an enzyme known as guanylyl cyclase. For example, NO causes vasodilation following a rise in Ca$^{2+}$ concentration due to ligand binding (i.e., acetylcholine, bradykinin, histamine, or insulin) to a specific receptor or shear stress in the blood vessel. Binding of Ca$^{2+}$ to calmodulin and subsequent binding to and activation of eNOS stimulates NO production. NO then diffuses across the endothelial cell membrane to a smooth muscle cell where it binds to and activates soluble guanylyl cyclase (sGC), which makes cGMP. In turn, cGMP activates protein kinase G (PKG), which phosphorylates a variety of channels and receptors. Activation of these channels and receptors leads to
inhibition of Ca$^{2+}$ influx into the smooth muscle cell, thus decreasing contraction and causing vasodilation. cGMP levels are regulated by sGC activity, activated by NO binding, and phosphodiesterases, particularly cGMP-specific phosphodiesterase 5, which hydrolyzes cGMP to 5'-GMP resulting in termination of the vasodilation signal.

Contrastingly, iNOS from macrophages is either present and functional or absent. It is induced by cytokines and interleukins upon immune challenge and is responsible for the major portion of NO production in mammalian systems. While it is an important function to produce cytotoxic agents, such as peroxynitrite, for the destruction of bacterial, fungal and cancerous cells, the overproduction of NO by this isoform is also responsible for endotoxic shock marked by a precipitous drop in blood pressure that can be life-threatening.

Nitric oxide synthases are prime examples of redox enzymes with newly discovered functions involving cellular signaling. The hierarchy of controlling factors involved in the function of these enzymes makes them extremely important targets for drug design, as a number of pathologies are associated with abnormal NOS regulation.

Selected References


Figure 4.6 Linear arrangement of the flavin- and heme-containing domains of all three nitric oxide synthases.
Figure 4.7 The formation of stable neutral blue semiquinone forms by reoxidation of flavin hydroquinone by artificial electron acceptors (a) or molecular oxygen (b).

\[
\begin{align*}
\text{NADPH} + \text{FAD/FMN} & \rightarrow \text{FADH}_2/\text{FMN} + \text{NAD}^+ \rightarrow \text{FAD/FMNH}_2 \\
\text{FAD/FMNH}_2 + \text{Fe}^{3+} & \rightarrow \text{FAD/FMNH} + \text{Fe}^{2+} \\
\text{NADPH} + \text{FAD/FMNH} & \rightarrow \text{FADH}_2/\text{FMNH} + \text{NAD}^+ \\
\text{FADH}_2/\text{FMNH} & \rightarrow \text{FADH}_2/\text{FMN} \\
\text{FADH}_2/\text{FMN} + \text{Fe}^{3+} & \rightarrow \text{FADH}_2/\text{FMNH}_2 \\
\text{FADH}_2/\text{FMNH} & \leftrightarrow \text{FADH}_2/\text{FMNH}_2 \\
\end{align*}
\]
**Figure 4.8** Structures of the heme and reductase domains of rat nNOS. A) The heme domain structure was generated from the PDB file 2G6K. Heme, NO and the axial Cys415 ligand are shown in ball-and-stick representation in yellow, blue and red respectively. Tetrahydrobiopterin and arginine are shown in stick representation in yellow and blue respectively. The Zn ion is shown as a black sphere. B) The reductase domain was generated from the PDB file 1TLL. FMN, FAD and NADP are shown in ball-and-stick representation in red, blue and yellow respectively.
4.3 Redox Regulation of Genes

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A number of studies have shown that the oxidative burst, induced by stress or developmental cues as well as alteration of antioxidant defenses is associated with specific changes in gene expression. There have been an increasing number of reports where gene/protein expression is influenced by cellular redox status and redox changes. Modification of specific amino acid residues of proteins is fundamental for modulation of gene expression. In particular, modulation of the redox states of cysteine residues has emerged as a widespread mechanism in cell regulation and signaling pathways. Cysteine residues play ubiquitous roles in mediating cellular responses to redox status through their ability to both detect changes in the redox environment and transduce a change in protein structure and function. Emerging evidence has also shown that several transcription factors are modulated by redox regulated DNA binding activity. In addition, mitogen activated protein kinase (MAPK) pathways not only represent cross talk between redox-mediated signaling and protein phosphorylation cascades, but also as a prime example of ROS activation for induction of signal transduction pathways.

4.3.A MAP kinase/cell cycle

A number of important signaling pathways are activated when cells are treated with oxidants. The association between MAPK signaling and oxidative stress is well documented. The MAPKs are modules of three successive protein kinases that are central for regulation of many cellular processes and are modulated, in part, by redox regulation. Depleting cellular GSH causes transient increases in ROS leading to the activation of ERK2, a classic MAPK. This activation is prevented by N-acetyl cysteine (NAC) treatment. Stress-activated protein MAP kinases (SAPK) are also sensitive to redox modulation. Activation of p38 SAPK by tumor growth factor beta requires free radical production. Similarly, protein phosphatases, particularly protein tyrosine phosphatases, need to be tightly controlled for maintenance of mitogenic signaling. Under basal conditions, ROS levels are low and protein tyrosine phosphatase activity is high;
following growth factor stimulation, ROS increases leading to transient inactivation of protein tyrosine phosphatases, and a concomitant burst of kinase activity, until ROS returns to basal levels and protein tyrosine phosphatase activity is restored. Both protein tyrosine phosphatases (and serine/threonine phosphatase) are redox-sensitive. The active sites of protein tyrosine phosphatases include cysteine and arginine residues separated by five amino acids, creating a low pKa, rendering cysteine residues more susceptible to oxidation. Oxidation of the cysteine residue results in protein tyrosine phosphatase inactivation. Several oxidative stressors, including $O_2^•−$, $H_2O_2$ and peroxynitrite, activate receptor tyrosine kinases following downstream signaling including MAPK.

The multifunctional p53 tumor suppressor gene encodes a transcription factor involved in cell cycle arrest. Reduced cysteines are essential for p53 DNA binding activity; mutations involving these residues are associated with many cancers. Several studies have shown that the cell cycle can be arrested in response to ROS and/or reactive nitrogen species (RNS). Alteration of cellular redox state by GSH depletion (increasing ROS) results in delayed progression through G1 and S phases as well as G2 arrest; thus cycling ceases or can induce programmed cell death.

4.3.B Redox control of gene expression

Among the possible targets of redox signaling pathways are redox regulated transcription factors. Redox alteration of transcription factor activity can occur via: (i) oxidative modification of the DNA-binding motif of the transcription factor by ROS, or (ii) a phosphorylation/dephosphorylation switch as a result of redox-regulated signaling. It is known that the DNA binding activity of a number of transcription factors is abolished by oxidizing conditions in vitro. Zinc finger motifs, a common DNA-binding domain, are particularly sensitive to redox changes; the thiol group of cysteine residues in the zinc finger protein Sp1 confers redox sensitivity (Figure 4.9).

Oxygen sensing in bacteria offers a number of particularly instructive examples illustrating the importance of the redox state for cellular metabolism and maintaining redox homeostasis. Bacteria have developed specific sensors that detect redox imbalance and trigger gene expression to generate an appropriate cellular response. For example,
recent work with the human pathogen *Staphylococcus aureus* has shown that a global regulator, MgrA, is both an important virulence determinant and functions as an oxygen sensor to regulate antibiotic/oxidative stress resistance. Using a genetic approach with transposon mutants, pathogenicity–related functions for MgrA were established. To elucidate regulatory mechanisms, the crystal structure for MgrA was solved. A potentially key cysteine residue was identified and experimentally tested. Oxidation of this cysteine regulates MgrA binding to DNA. MgrA DNA binding normally occurs under oxidative stress; MgrA binding induces several pathways regulating antibiotic resistance as well as other defense related genes. Thus, these bacteria utilize a single protein that is oxidatively regulated, to activate a number of stress responsive pathways that serves as a general strategy for responding to numerous environmental cues involving oxidative stress.

4.3.C Peptide editing and thiol mediated redox regulation

The mechanism for distinguishing and selecting peptides by MHC class 1 molecules during antigen processing is a result of thiol-based redox regulation. This was determined from mechanistic studies evaluating how MHC class 1 molecules select peptides from a complex background of self vs. non-self proteins in the ER. Peptide loading complexes were purified and components identified by tandem mass spectrometry. Among the proteins identified, protein disulfide isomerase, was established as a member of the antigen processing loading complex. This enzyme is necessary for the correct formation of disulfide bonds and is involved in disulfide bond oxidation and reduction as well as isomerization. Functional studies determined the specificity of the protein disulfide isomerase in this complex and the role for this enzyme MHC class 1 antigen presentation. RNA knockdown of protein disulfide isomerase demonstrated the physiological relevance of this enzyme in MHC class 1 processes. Coupling the enzymatic function of protein disulfide isomerase and the knockdown experiments, suggested that redox regulation of MHC class 1 molecules is important. It is believed this redox switch might provide a checkpoint for protein quality control of complex assembly,
and permit rapid and sustained responses to pathogens as well as deterring unwanted immune responses.

It is evident that cells employ redox status to regulate wide variety of physiological functions. Although there are gaps in our knowledge of ROS and signaling, it is known that ROS are produced continuously in the cell during normal growth and development. All cells harbor an efficient suite of antioxidant systems that protect against perturbations that increase ROS (see Chapter 2). ROS can propagate signals to multiple subcellular pathways thus influencing a variety of cellular processes (Figure 4.10). Since the cell has an efficient anti-ROS system, how do basal (non-toxic) concentrations of ROS exist? This is an issue often faced with signaling and gene regulation. Possibly basal ROS and the antioxidants are located in different cellular compartments, thus enabling ROS to affect its target prior to being affected by the cells antioxidant system.

The sensitivity of reactive cysteine residues to oxidation builds redox switches into proteins that can respond to changes in cellular redox status. The thiolate anion can be stabilized by proximity to hydrogen bond donors, basic residues and metal ions. Thus, the reactivity of cysteine residues towards ROS can influence the selectivity of the response. The capacity of redox-sensitive cysteine residues to both detect and affect a response to changes in redox status provides a relatively simple and rapid mechanism to elicit regulation of biological responses.

Selected References

Figure 4.9 Crystal structure of Zif268 protein-DNA complex (from RCSB PDB 1AAY) showing thiol groups (yellow) and Zn$^{2+}$ (red). Blue $\gamma$-helices represent the zinc fingers with the GGCGT nucleic acid binding finger in the lower left corner.
Figure 4.10 Generalized view of ROS mediated signal transduction. At low concentrations, ROS can indirectly trigger signaling leading to proliferation, differentiation or death (apoptosis). At high concentrations, excess ROS leads directly to death (necrosis).
Multicellular organisms eliminate redundant, damaged, or unnecessary cells by a gene-directed death process. Programmed cell death or its morphological equivalent, apoptosis, is a genetically encoded cellular suicide that is essential for normal growth, development and tissue homeostasis in all multicellular eukaryotes. In humans and other animals, dysregulation of this natural cell death pathway significantly contributes to a number of major diseases. Defects of genes that control death pathways that save cells normally destined to die can underlie both cancer and autoimmune diseases, while defects that promote cell death that do not normally occur can contribute to stroke, AIDS, Alzheimer’s, Parkinson’s and other neurodegenerative diseases. In addition, most viruses and intracellular bacteria, as well as plant pathogenic fungi control the cell death pathways in the host cells, thus linking apoptosis to infectious diseases.

Morphological and biochemical features associated with this cell death process include cell shrinkage, membrane blebbing, chromatin condensation, DNA cleavage and fragmentation (resulting in a characteristic “ladder”), and externalization of the inner membrane lipid phosphatidylserine. A group of dedicated cysteine directed proteases termed caspases, are activated during this process and cleave major structural proteins during the orderly dismantling of the cell. These pro-apoptotic caspases are present as inactive zymogens and are induced and activated following an apoptotic stimulus. These proteases are divided into two groups: initiator or upstream caspases and executioner caspases. The net result of apoptosis is the clean removal of cells without an inflammatory response, which is in contrast to necrotic or accidental cell death where the cell does not actively participate in the process, and inflammation occurs.

4.4.A Apoptotic pathways

In mammalian systems, apoptosis proceeds by at least two major pathways, extrinsic and intrinsic (Figure 4.11). As is often the case with complex signaling pathways, the lines of distinction between these “independent” pathways can be obscured by cross-talk. The extrinsic or death receptor pathway is typified by members of the
tumor necrosis factor family of receptors. Following binding of specific “death” ligands, receptors aggregate, forming a death-inducing signaling complex that results in the activation of a caspase 8, an initiator caspase. Caspase 8 then proteolytically activates downstream “executioner” caspases (e.g. caspase 3, 6, 7), which cleave structural and other proteins for the orchestrated dismantling of the cell.

The intrinsic pathway can be triggered by numerous stimuli, including growth factor withdrawal, DNA damage and oxidative stress. This pathway generally involves mitochondria, which when stimulated by an apoptotic signal, releases several apoptotic regulators, including cytochrome c and other proteins normally sequestered between the inner and outer mitochondrial membranes. Upon release, cytosolic cytochrome c binds to an adaptor protein (Apaf-1), resulting in the dATP-dependent formation of the “apoptosome” comprised of oligomers of cytochrome c, Apaf-1 and procaspase-9. This high molecular weight complex results in the proteolytic activation of caspase 9. Caspase 9, in a manner similar to caspase 8, activates downstream caspases for cellular demise.

4.4.B Reactive oxygen species and apoptosis

Many redox-sensitive proteins are involved in regulating apoptotic pathways suggesting that the redox environment of the cell is important. The production of ROS, in particular, has been associated with programmed cell death in many pathological contexts including stroke, inflammation, ischemia, lung edema and neurodegeneration. Several chemical and physical treatments capable of inducing apoptosis are also known to generate oxidative stress. The major physiological source of ROS in mammals is the mitochondrion where oxygen is reduced to water. A crucial event associated with the intrinsic pathway is the uncoupling of oxidative phosphorylation in the mitochondria and the dissipation of mitochondrial transmembrane potential, a decrease in ATP and an increase in ROS. The Bcl-2 family of apoptotic regulators contains both pro and anti-apoptotic proteins that localize to the outer mitochondrial membrane. The sensitivity of cells to apoptotic stimuli often is dependent on the balance between pro- and anti-apoptotic Bcl-2 family member proteins. Under conditions of oxidative stress, cytoprotective proteins like Bcl-2, prevent the ability of ROS to induce apoptosis.
Conversely, Bad and Bax, pro-apoptotic Bcl-2 family members, act as sensors of cellular damage, translocate to the mitochondria following such stress, increase the generation of ROS and disrupt the ability of anti-apoptotic proteins to inhibit death; caspases are activated and death ensues. The resultant phenotypes are concentration dependent; at relatively low levels, ROS function as signaling molecules promoting proliferation and survival whereas higher levels of ROS are apoptotic while even higher levels are necrotic. ROS mediated apoptosis causes disruption of the mitochondrial membrane potential and permeability transition leading to cellular dysfunction; ATP synthesis is blocked, redox molecules including NADH, NADPH, and GSH are oxidized, and ROS levels increase. In the death receptor pathways, ROS accumulates prior to all morphological and biochemical alterations associated with apoptosis. Antioxidant treatments prevent death receptor mediated apoptosis.

In most cases, ROS triggers programmed cell death by oxidatively altering cellular proteins and other components or by directly activating the mitochondrial pathway. Apoptotic effectors, particularly caspases, are redox-sensitive. All caspases harbor a QACXG motif, which contains the active site cysteine. This cysteine residue is sensitive to redox changes. Both thioredoxin and GSH are required for caspase-3 activity and in regulating apoptosis signal-regulating kinase-1 (ASK-1), a mitogen active protein kinase kinase kinase (MAPKKK) that regulates the activation of c-Jun N-terminal kinase (JNK) that is involved in stress signal transduction pathways, including apoptosis. Cells therefore require a reducing environment for caspases to function properly. ASK-1 is a redox sensor kinase involved in oxidative stress-induced activation of JNK, which leads to apoptosis. Thioredoxin has been shown to be a negative regulator of the ASK-1/Jnk pathway; thus functioning essentially as a cytoprotectant. JNK is maintained at low levels in non-stimulated cells, and its enzymatic activity is inhibited by interaction with glutathione S-transferase. Following apoptotic stimuli, such as H$_2$O$_2$ or UV radiation, this complex dissociates and JNK is activated via phosphorylation by ASK-1. The identification of thioredoxin and glutathione S-transferase as modulators of ASK-1 and
JNK suggests a mechanistic model for how redox–mediated signaling events can result in programmed cell death (Figure 4.12).

Other important factors contributing to redox regulation of apoptosis include heat shock proteins (Hsps). Hsps can directly interact with various apoptotic proteins at key points along the pathway. Hsps have anti-apoptotic functions by providing time for cellular adaptation during conditions that are otherwise lethal. For example, several apoptotic stimuli induce Hsp27 overexpression. Hsp27 can be cytoprotective in several ways including decreasing ROS levels and neutralizing the toxic effects of oxidized proteins. Hsp70 can inhibit caspases and prevent oligomerization of Apaf-1. Control of programmed cell death by Hsps is dependent on the nature of the stimulus and cell type.

In terms of reactive nitrogen species, NO can function as a cell killer or protector depending on its concentration. At relatively high concentrations, NO can induce programmed cell death or necrosis. The cytotoxicity of NO is primarily due to interaction with $\cdot O_2^*$ to form peroxynitrite. At relatively low concentrations, NO inhibits programmed cell death due to its reactivity with thiol groups. NO can block catalytic cysteine residues by S-nitrosylation of caspases and can also increase the concentration of Bcl-2, thus indirectly preventing programmed cell death at several points in the pathway.

Due to its high concentration, the GSH:GSSG redox couple usually mirrors the intracellular redox environment. The concentration of GSH and/or the ratio of GSH/GSSG) are crucial in processes leading to programmed cell death under conditions of oxidative stress. For example, the release of cytochrome c, a major component of the intrinsic cell death pathway mentioned previously, is significantly affected by a decrease in GSH.

While the precise mechanisms by which ROS regulates apoptosis is not entirely clear, modification of important cysteine residues may account for regulation of the redox balance in cells undergoing programmed cell death. Cysteine redox status can influence protein structure leading to an activation or inhibition of function. Antioxidants such as superoxide dismutase, catalase and GSH peroxidase also influence redox status by
scavenging ROS. Although questions remain, redox regulation of oxidative stress is central to the modulation of programmed cell death.

Selected References


Figure 4.11 Major apoptotic pathways in mammalian cells. Pro-apoptotic proteins include caspases, Bak, Bic, Bax, cytochrome, Apaf-1 and Smac. Anti-apoptotic proteins include IAPs, Bcl-2, and Bcl-xl.
Figure 4.12 Oxidative stress induced apoptosis and MAP kinases. ASK-1, a MAPKKK is activated by ROS. Activated ASK-1 induces kinase activity of JNK and/or p38 SAPKs. Several antioxidants (enzymes, heat shock, protein) negatively regulate kinase activation. SAPK activation phosphorylates several downstream targets including the transcription factor c-Jun or p53 which indirectly or directly cause programmed cell death. SAPKs appear to also affect oxygenation of Bcl-2 family members, inhibiting Bcl-2 and activating Bax.
4.5 Metal Homeostasis
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The inorganic chemistry of metals is widely utilized in various biological processes such as enzyme reactions, signal transductions, electron transfer, and oxygen transport. Transition metal ions in particular play critical roles as electron transfer intermediates in various redox reactions. Organisms must acquire metals from the environment and incorporate them into metalloproteins by the post-translational addition of metal or metal-containing prosthetic groups. However, excess metal accumulation and their release in free reactive forms can be toxic. Since both deficiency and excess lead to serious problems in organisms, regulation of metal metabolism, including uptake, trafficking, assembly into metalloproteins, and detoxification are clearly important. Recent progress in elucidating mechanisms for metal homeostasis are revealing underlying principles of metal metabolism and implicating metals in development, growth, and disease. Since disorders in metal metabolism are linked to a number of health problems, studies on metal metabolism can have important clinical implications. This section will summarize the fascinating mechanisms for metabolism of copper (Cu) and iron (Fe) in mammals and the implication of defects in metal metabolism in disease.

4.5.A Physiological significance of metal metabolism

Redox-active metals mediate electron transfers in various biochemical reactions. The catalytic centers of many enzymes contain Cu, Fe, heme or iron-sulfur clusters that are essential for function. For example, energy generation by mitochondrial oxidative phosphorylation depends on Cu and heme incorporation into proteins, such as
cytochrome c oxidase. Cu and zinc-containing superoxide dismutase (Cu, Zn SOD) utilizes Cu in the detoxification of $O_2^\cdot$ (see Chapter 3.1.B). Aconitase in mitochondrial citric acid cycle is an example of an Fe-S center enzyme. Cu-containing enzymes play essential roles in the synthesis of catecholamine, a neurotransmitter. Furthermore, hemoglobin in red blood cells carries a major portion of Fe in mammals, and oxygen transport by heme in red blood cells is essential for respiration. Nutritional metal deficiency and genetic diseases of metal metabolism have further provided striking evidence that metals are critical trace elements in a number of other physiological processes.

While metals are essential nutrients, their excess accumulation is toxic. Transition metal ions readily catalyze reactions that result in the production of hydroxyl radicals through the Fenton and Haber-Weiss reactions (reactions 4.1 & 4.2).

\[
\begin{align*}
\text{Cu}^{+}(\text{Fe}^{2+}) + \text{H}_2\text{O}_2 & \rightarrow \text{HO}^- + \text{HO}^- + \text{Cu}^{2+}(\text{Fe}^{3+}) \\
\text{O}_2^\cdot^- + \text{Cu}^{2+}(\text{Fe}^{3+}) & \rightarrow \text{Cu}^+(\text{Fe}^{2+}) + \text{O}_2
\end{align*}
\]

[4.1] [4.2]

The highly reactive oxygen intermediates are responsible for lipid peroxidation, oxidation of proteins, and cleavage of nucleic acids. These cellular damages are believed to be major contributing steps to various diseases in humans.

4.5.B Metal uptake from the extracellular environment

Organisms obtain metals from the diet and the environment through specific transporters at the cell membrane. Although Cu and Fe are abundant in the earth’s crust, these metals are not readily bio-available due to limited solubility. Cell surface metalloreductases containing two hemes as prosthetic groups for transmembrane electron
transport have been identified in yeast and plant. They play critical roles in enhancing uptake of oxidized metals. Although a potential mammalian Fe reductase has been proposed, its role in Fe absorption has not been firmly established. The gene encoding Cu reductase remains to be identified.

Several high and low affinity Cu and Fe membrane transport systems have been characterized. The Ctr1 (copper transporter 1) family of proteins, conserved in eukaryotes from yeast to mammals, is generally accepted as a Cu transporter (Figure 4.14). Members of the Ctr1 family have no apparent homology to known proteins. Although the overall sequence similarity between yeast Ctr1 and human Ctr1 is low, Ctr1 family members have common structural features. In fact, both human and plant Ctr1 function in yeast cells. This suggests that the mechanism of Ctr1-mediated Cu transport is conserved among eukaryotes. Evidence that Ctr1 is critical for cellular Cu transport is accumulating. For example, Ctr1 overexpression stimulates cellular Cu uptake, and Ctr1 gene knockout in yeast and mice reduces Cu levels. However, its precise role in Cu transport is not currently defined. It is uncertain whether Ctr1 is a Cu transporter, a Cu receptor, or a component of Cu transporter.

The mammalian Fe uptake system is comprised of several components, including Fe-binding transferrin in serum, cell membrane transferrin receptors, and Fe transporter (DMT1/DCT1/Nramp2) (Figure 4.15). Transferrin-bound transferrin receptor undergoes endocytosis. The acidic environment in the endosome causes Fe to be released from transferrin, and Fe is transported to the cytoplasm through DMT1 by a proton co-transport mechanism. DMT1 also plays a role in non-heme Fe uptake at the intestine.
Since oxidized Fe forms insoluble complexes, Fe$^{3+}$ reductase is required for Fe uptake at the intestine. Dcytb, a cytochrome b-like protein, was identified as an Fe reductase. However, gene knockout in mice revealed that Dcytb is not essential for intestinal non-heme Fe absorption. Thus, an alternative Fe reductase must exist, since Fe reduction appears to be an essential step for Fe absorption from the diet. Dietary heme is also a significant source of Fe for humans and carnivorous animals and a heme transporter has been cloned. Several other Fe uptake systems that are specific for substrate, cell, or developmental stage have been identified in mammals and other eukaryotes.

4.5.C Intracellular metal distribution by target-specific chaperones

Once metals are transported into the cell, they must reach their appropriate destination efficiently and are incorporated into metal-requiring proteins without participating in harmful side reactions. The mechanism for cytoplasmic Cu distribution to Cu-requiring proteins or to cellular compartments by target-specific Cu carrier molecules (Cu chaperones: Atx1/Atox1, Cox17, and CCS) have been identified (Figure 4.14). Atx1 delivers Cu to the secretory compartment where Cu is loaded into Cu-containing proteins. Another Cu chaperone, Cox17, has been shown to play an essential role in the delivery of Cu to the mitochondria. The delivery of Cu to cytoplasmic Cu,Zn SOD is mediated through a soluble factor identified as CCS that directly interacts with Cu,Zn SOD to transfer Cu. Since free Cu is toxic and virtually no free Cu appears to be present in the cell, it is reasonable to predict that the Cu chaperones acquire Cu directly from a plasma membrane Cu transporter. However, although it has been demonstrated that Ctr1-mediated Cu uptake is necessary for Cu distribution by chaperones, the interaction
between Ctr1 and Cu chaperones has not been demonstrated. Cu chaperones may transiently bind with Ctr1, or there may be other components that are required for the Cu relay between Ctr1 and Cu chaperones. Chaperone-mediated intracellular Cu distribution is a new concept in metal metabolism. Most intracellular Fe is moved to the mitochondria for the synthesis of heme and Fe-S clusters. It is unlikely that free Fe is released in the cytoplasm following uptake from the outside of the cell. However, specific carrier molecule(s) that transfer Fe to the mitochondria have not been identified.

4.5.D Subcellular membrane metal transporters

The Menkes (MNK, ATP7a) and Wilson (WND, ATP7b) disease genes encode P-type ATPases localized in the trans-Golgi network where they deliver Cu to proteins that traverse the secretory pathway (Figure 4.14). The observations of Cu hyper-accumulation in MNK-defective fibroblasts and intestinal epithelial cells and the trafficking of MNK protein to the plasma membrane under conditions of high Cu indicate that MNK plays a role in cellular Cu efflux. The MNK gene is strongly expressed in many organs and tissues, including intestinal mucosal cells, but only in a trace amount in the liver. WND expression is seen almost exclusive to hepatic and brain tissue. This expression pattern is consistent with the requirement of WND for incorporation of Cu into ceruloplasmin, an Fe oxidase, in the liver and for biliary excretion of Cu. Mitochondria represent another membrane-bound compartment into which Cu needs to be transported for incorporation into cytochrome c oxidases. It is now apparent that Cu homeostasis in the mitochondrion is considerably more complex than previously thought. Recently, the role of the Cu chaperone, Cox17, as the main mitochondrial Cu shuttle has come into question. Cox17,
tethered to the mitochondrial inner membrane by fusion with the N-terminal transmembrane domain of an inner membrane protein, complimented the respiratory deficiency of ∆cox17 cells. Thus, the role of Cox17 appears to be confined to the mitochondrial intermembrane space where Cu(I) is translocated to the inner membrane proteins Sco1 and Cox11 that play critical roles for incorporation into the Cu_A and Cu_B sites of cytochrome c oxidase, respectively. Evidence also exists for a non-protein pool of Cu in the mitochondrial matrix. If Cox17 does not supply Cu to the mitochondria, then how does it get there? One possibility is that Cu transporters exist within the mitochondrial outer and inner membranes. Such transporters have remained elusive.

Mitochondria are the major organelles utilizing Fe for the synthesis of heme and Fe-S clusters. However, the mechanisms for uptake and export of Fe in the mitochondria and regulation of these steps are not well understood. This is a key question that remains to be answered in cellular Fe metabolism.

4.5.E Heme and iron-sulfur cluster synthesis

Heme in hemoglobin of red blood cells is a major form of Fe in mammals. The pathway for heme biosynthesis, including the roles and compartmentalization of the enzymes, has been established. Fe-S clusters are also important Fe-containing prosthetic centers that are essential for electron transport, enzymatic catalysis, and gene regulation. The scaffolding proteins required for the assembly of Fe-S cluster has been identified. The mechanisms of action of these proteins need to be fully elucidated to understand the molecular details of Fe-S cluster assembly. Synthesis and export of Fe-S clusters from the mitochondria play important roles in cellular Fe homeostasis. It has been proposed that
cytoplasmic Fe-S cluster levels are sensed by a regulatory system to control Fe import
into mitochondria and Fe homeostasis.

4.5.F Cellular storage

Excess intracellular Cu and Fe are sequestered in cells. The biological
significance of this mechanism appears to be both in detoxification and storage.

Metallothionein is a well-characterized chelator of various metals such as Cu, Zn, and
Cd. Mammalian metallothionein is a 60-61 amino acid peptide containing 20 cysteines.

Metal reconstitution in vitro demonstrated that metallothionein coordinates up to 12
atoms of Cu through thiolate bonds to cysteine residues. Sensitivity to metal toxicity in
metallothionein gene disruption in mice and in yeast strongly supports a role for
metallothionein in metal detoxification. However, it is not clear whether metallothionein-
bound Cu plays a significant role in Cu storage. The yeast vacuole is involved in
sequestration of metals, and metal-specific transporters (e.g., Ctr2 Cu exporter, CCC1 Fe
importer, Fth1 and Fet5 Fe export complex) at the vacuolar membrane have been
identified. Ferritin is an Fe storage multimeric protein that is composed of 24 light and
heavy chain subunits (Figure 4.15B) that sequesters up to 4500 Fe atoms in the
ferrihydrite form. Ferroxidase activity of the H subunit is required for Fe sequestration.

Various stimuli, including cellular Fe deficiency, degrade ferritin to mobilize stored Fe.

4.5.G Metal export

Cu-dependent trafficking Cu-transporting P-type ATPase from the secretory
compartment to the plasma membrane is an important step for Cu export. This appears to
be particularly important for Cu absorption from the intestine. WND Cu transporting
ATPase-mediated Cu excretion into bile from the liver is a major mechanism for systemic Cu homeostasis in mammals (Figure 4.14).

Ferroportin, an integral membrane protein, mediates Fe export at the basolateral membrane of enterocytes, which is critical for Fe absorption (Figure 4.15A). Ferroportin-mediated export of Fe from macrophages that engulf senescent erythrocytes also plays a critical role in recycling Fe (Figure 4.15C). The mechanism of Fe excretion from the body has not been identified. Only a minimal amount of Fe is excreted from the body through loss of hair and skin cells. It appears that systemic Fe homeostasis relies on regulation of Fe absorption from the diet and mobilization from intracellular storages.

4.5.H Regulation of metal metabolism

Regulation of metal homeostasis basically leads to maintenance of optimal levels of bioavailable metals in the cell, organ and whole organism. This is achieved by controlling the expression levels and/or localization of the components involved in metal uptake, distribution, storage, and excretion. Cu and Fe homeostasis is maintained by several regulatory mechanisms that include metal binding to transcriptional and post-transcriptional regulators, metal-mediated interactions between components involved in metal metabolism, communication between organs, and/or signals from metabolic pathways in which metals are required. Some of the systems are unique, providing new paradigms for gene regulation.

4.5.H1 Transcriptional regulation

With the exception of the metallothionein gene, transcriptional regulation of genes encoding components of Cu metabolism has not been characterized in mammalian
cells. However, transcriptional regulation is a major mechanism for Cu homeostasis in yeast. The Cu-sensing Mac1 transcription regulator plays a critical role in Cu homeostasis in yeast by regulation of transcription of the genes encoding Cu uptake proteins. Ace1 is another transcription factor that responds to toxic levels of Cu to regulate the Cup1 gene encoding metallothionein. Both regulators appear to bind Cu directly, which controls their activities.

Aft1 and Aft2 regulate Fe metabolism in yeast. The two transcription factors have significant sequence homology with each other. Interestingly, they have overlapping but independent functions in their target gene regulation. The mechanisms of sensing Fe status by Aft1 and Aft2 have not been elucidated, but Fe-S cluster levels in cytoplasm are implicated in Aft1/2 regulation. In mammals, cytokines (e.g., TNF-α, interleukin, IFN-γ) regulate expression of several components of Fe metabolism (e.g., ferritin, transferrin receptor 1, ferroportin, hepcidin). Cytokine-induced regulation of Fe metabolism seems to be linked to host defense mechanism by limiting availability of Fe to invading pathogens.

4.5.H2 Post-transcriptional regulation

Iron regulatory protein (IRP) 1 and 2 play major roles in the regulation of expression of proteins involved in Fe metabolism (Figure 4.17A). IRP binds to an iron-response element (IRE) which is a conserved hairpin structure in the untranslated regions (UTR) of mRNAs encoding proteins involved in Fe metabolism (Figure 4.17B). IRP binding to IRE located at the 5’ UTR inhibits translation of target mRNAs encoding ferritin, mitochondrial aconitase, ferroportin, and aminolevulinic acid synthase, the first
enzyme in the heme synthesis pathway. The 3’ UTR of transferrin receptor 1 carries multiple IREs. Binding of IRP to the 3’ UTR enhances mRNA stability and leads to increased translation of transferrin receptor 1. IRP1 is a cytoplasmic Fe-S cluster-containing aconitase that is converted to an mRNA binding protein depending on cellular Fe status and other signals that perturb the Fe-S cluster in IRP1. For example, reduced Fe-S cluster synthesis resulting from Fe deficiency leads to defective Fe-S cluster assembly in IRP1. The Fe-S cluster of the IRP1 is also sensitive to reactive oxygen and nitrating species (e.g., H$_2$O$_2$, NO). The Fe-S cluster-defective form of IRP1 loses aconitase activity and serves as an IRE binding regulator. Thus, Fe deficiency inhibits translation of Fe storage molecules, mitochondrial citric acid cycle, Fe export, and heme synthesis. At the same time, binding of IRP to the 3’ UTR of transferrin receptor 1 stabilizes the mRNA and enhances its translation, which leads to increased Fe uptake into cells. Fe transport and Fe-S cluster incorporation to IRP1 converts it to aconitase. This is a feedback mechanism for controlling IRP1 activity. The mode of regulation of IRP2 by Fe is different from that of IRP1. Fe stimulates the proteosomal degradation of IRP2. It appears that direct binding of Fe to IRP2 is a signal for its degradation. IRP gene knockout in mice suggested that IRP2 dominates the Fe-deficiency responses in mice. It is not clear yet why mammals utilize two IRPs in Fe regulation and whether the IRPs have distinct role(s) in Fe homeostasis.

It was recently discovered that a Fe-responsive post-transcriptional regulatory process controls metabolic reprogramming in yeast. Under Fe deficiency, yeast Cth2 protein specifically down regulates mRNAs encoding proteins that play roles in many Fe-
dependent physiological processes. mRNA turnover requires the binding of Cth2 to specific AU-rich elements in the 3’ UTRs of genes targeted for degradation. Identification of Cth2 target genes suggested that Cth2-mediated acceleration of mRNA decay could be an important mechanism for coordinated remodeling of cellular metabolism during Fe deficiency.

4.5. H3 Post-translational regulation

Cu-mediated regulation of trafficking and turnover of Cu transporters have been characterized (Figure 4.16). In low Cu medium, the human MNK (ATP7a) and WND (ATP7b) are localized in the trans-Golgi network where they transport Cu for its incorporation into secretory proteins. When Cu levels are elevated, MNK is mobilized to the plasma membrane in a reversible manner. The biological significance of this trafficking event is that MNK may participate in Cu extrusion at the plasma membrane to export Cu from intestinal cells to blood for circulation and to protect cells from Cu toxicity in other organs. Excess Cu mobilizes WND from the trans-Golgi network to a cytoplasmic vesicle-like compartment in the liver. This appears to be an important mechanism for excretion of excess Cu into bile to maintain systemic Cu homeostasis. While the regulation of Cu excretion is relatively well characterized in mammals, Cu-induced regulation of Cu absorption has been reported only recently. Elevated extracellular Cu triggers the endocytosis as well as degradation of human Cu transporter Ctr1. Further studies will define the physiological significance of the modes of Ctr1 regulation in Cu metabolism.
Several lines of evidence have demonstrated that hepcidin is a master regulator of systemic Fe homeostasis (Figure 4.17C). Systemic Fe status, inflammation and hypoxia regulate hepcidin gene expression. Hepcidin (84 amino acids) is secreted from the liver and is then cleaved to small peptides (20, 22, or 25 amino acids) that circulate in blood and are excreted in urine. Recent studies showed that hepcidin regulates cellular Fe efflux in the intestine, and from macrophages and hepatocytes by binding to ferroportin and induces its internalization and degradation. Consequently, this post-translational regulation of ferroportin by hepcidin completes the Fe regulatory loop in mammals.

4.5.1 Genetic disorders in metal metabolism

Consistent with the essential roles of Cu and Fe and their complex metabolism, many of the proteins involved in Cu and Fe homeostasis have been linked to human genetic disorders. X-linked Menkes disease and autosomal recessive Wilson disease are well-characterized genetic diseases of Cu deficiency and toxicity, respectively. Patients with Menkes disease die in the first year of life due to defects in the function of Cu-requiring enzymes. The onset of Wilson disease in humans is variable as is the severity of symptoms, which suggests environmental and/or genetic factors modulate progression of the disease.

Although the Ctr1-mediated Cu uptake system and intracellular Cu chaperones have been identified, no genetic disorders have been linked to defects in Cu uptake and cytoplasmic distribution. Recent studies on knockout mice lacking the genes encoding Ctr1, Cox17, or Atx1 demonstrate their critical role in embryonic development. Thus, it
is possible that humans possessing non-functional Ctr1 may die during development in utero.

Hereditary hemochromatosis is the most common inherited disease among Caucasians. It is estimated that as many as one in eight individuals of Northern European descent are carriers of a faulty allele. Hereditary hemochromatosis is characterized by Fe overload, resulting in cirrhosis of the liver, diabetes, and heart failure, presumably due to Fe toxicity in the organs. Regulation of Fe absorption at the intestine is defective in hereditary hemochromatosis patients. Four different types of hemochromatosis based on the mutated gene, have been characterized: 1) missense mutations in the HFE gene encoding an atypical major histocompatibility class I protein is linked to type 1 hemochromatosis. Although the function of HFE is not known, it forms a complex with transferring receptor 1 and inhibits transferrin binding. This interaction between HFE and transferring receptor 1 leads to reduction of cellular Fe uptake; 2) HFE2 gene mutation is linked to type 2 hemochromatosis. HFE2 may play a role in regulation of hepcidin expression; 3) transferrin receptor 2 mutations that disrupt complex formation with HFE lead to type 3 hemochromatosis; and 4) heterozygosity for mutations in ferroportin leads to an autosomal dominant type 4 hemochromatosis. Interestingly, ferroportin forms a dimeric complex and the mutant forms interfere with plasma membrane trafficking of the wild type monomer and/or hepcidin-induced degradation of mutant and normal monomer complex at the plasma membrane.

Many other relatively rare Fe-related genetic diseases have been identified in humans and/or animals. Cerulopasmin is a Cu-containing ferroxidase in plasma that is
required for Fe export and binding to transferrin. Aceruloplasminemia patients exhibit Fe accumulation in cells such as hepatocytes and macrophages. Lack of transferrin in atransferrinemia patients induces severe defects in mobilization of Fe from stores for its utilization (e.g., in hemoglobin synthesis). This leads to hypoxia-induced up-regulation of Fe uptake from the intestine and excess Fe accumulation in Fe storage tissues. Defects in frataxin affect Fe-S cluster synthesis in mitochondria. Fe accumulation in mitochondria results in mitochondrial damage and progressive neurodegeneration.

4.5 J Perturbation of metal homeostasis and degenerative disorders

Aberrant Cu and/or Fe metabolism is implicated in multifactorial human disorders such as neurodegenerative diseases, cardiovascular diseases, and cancer. For example, Cu has been implicated in the etiology of Alzheimer’s disease, which is characterized by accumulation of β-amyloid (Aβ), a proteolytic product of the amyloid precursor protein (APP). APP binds Cu to reduce Cu(II) to the more reactive Cu(I). The binding of Cu to Aβ elevates Aβ aggregation. Cu is highly concentrated within senile plaques, the histopathologic hallmarks of Alzheimer’s disease that are generated by the deposition of Aβ. Deposition of Fe in the brain is also a common feature of neurodegenerative diseases such as Alzheimer’s disease and Parkinson’s disease. Although it is not known whether Fe and Cu deposition is a cause or consequence of these diseases, Fe and Cu toxicity likely plays an important role in progression of neuronal damage observed in these diseases.

Selected References


**Figure 4.13** Metal metabolism is comprised of many steps. (A-C) Metals are absorbed in the intestine from the diet and transported to the organs and tissues. Membrane metal transporters and carriers are involved in the absorption and transport steps. (D) Metal-specific transporters at the cell surface take up metals at the peripheral organs and tissues. (E) Cytoplasmic metal carrier molecules and metal transporters at the subcellular organelles play critical roles in intracellular metal trafficking, compartmentalization, and utilization. (F) Mechanisms for metal storage and mobilization have been characterized. (G) Metals accumulated in excess or non-physiological metals are excreted from the body. Expression, localization, and/or activities of the proteins involved in metal metabolism are regulated by several different delicate mechanisms to maintain optimal levels of metals in the body.
Figure 4.14 Copper transport and distribution. Ctr1, is an integral membrane protein that is an essential component of cellular copper uptake. Two Cu carriers (CCS, Atx1) in the cytoplasm deliver Cu in a target-specific manner. Copper-transporting CPX-type ATPases (MNK and WND) at the membrane of the post-Golgi compartment transport Atx1-delivered Cu for incorporation of Cu into secretory proteins. CCS directly interacts with Cu,Zn SOD for Cu relay. Cox17 plays a critical role in Cu insertion into cytochrome c oxidase.
Figure 4.15 Iron transport and distribution. (A) Intestinal Fe transport. DMT1 transports Fe at the apical side of the intestinal epithelial cell layer. HCP1 has been identified as a heme transporter. Iron exported by ferroportin is oxidized by hephaestin, a Cu-containing ferroxidase, and binds to transferrin (Tf). (B) Fe uptake by organs and tissues. The Tf and transferrin receptor (TfR) complex undergoes endocytosis. Fe is released from Tf in the acidic endosome and transported to the cytoplasm through DMT1. The majority of the Fe is utilized for the synthesis or heme and iron-sulfur (Fe-S) clusters. Excess Fe is stored in ferritin. (C) Fe mobilization from storage cells. Ferroportin exports Fe from hepatocytes and reticuloendothelial macrophages that engulf senescent red blood cells (RBC). Ceruloplasmin, a Cu-containing ferroxidase in serum, oxidizes Fe for incorporation into Tf.
Figure 4.16 Regulation of Cu metabolism. MNK localizes at the post-Golgi compartment and the plasma membrane depending on cellular copper levels. MNK exports copper at the plasma membrane. Copper-dependent localization of WND at the vesicles is implicated in excretion of excess copper into bile from the liver, which is an essential step for systemic copper homeostasis in mammals. Ctrl1 cell surface expression appears to be controlled by copper-dependent manner, which may be a critical mechanism for the regulation of copper uptake.
Figure 4.17 Regulation of Fe metabolism. (A) Regulation by iron regulatory protein 1 (IRP1) and iron regulatory protein 2 (IRP2). IRP1 is a cytoplasmic aconitase containing a 4Fe-S cluster. Lack of Fe-S cluster assembly into IRP1 due to Fe deficiency converts it to an iron regulator. Excess Fe induces IRP2 degradation, which controls cellular IRP2 levels. (B) Regulation of translation by Fe regulatory proteins. IRP1 and IRP2 bind to iron response element (IRE) in the 5’ or 3’ untranslated regions of their target mRNAs to control translation efficiency of their targets. (C) Regulation of Fe metabolism by hepcidin. Excess Fe levels and inflammation induce transcription of hepcidin, a systemic regulator of Fe metabolism. Hepcidin circulates in the blood and binds to ferroportin to induce its endocytosis and degradation. This reduces Fe absorption from the intestine and mobilization of Fe from storage proteins.
4.6 Redox Enzymology
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This section describes the study of redox enzymes, which either catalyze oxidation-reduction reactions or depend upon oxidation or reduction for activity. It excludes electron-transfer proteins, like cytochrome c or ferredoxin that transfer electrons but do not catalyze reactions. Some oxidation-reduction reactions involve the net uptake or donation of electrons (e.g., carbon monoxide oxidation), while others involve an internal redox reaction, for example, the uptake/release of an electron that is returned during the overall reaction.

All redox enzymes require a redox-active prosthetic group, either a metallocofactor or an organic cofactor (FAD, NAD$^+$, etc.). Since there are hundreds of redox enzymes, Table 4.1 cannot be exhaustive, but includes representative example covering most of the known redox cofactors. A collection of reviews describing the mechanisms of a number of redox enzymes is available.

Redox enzymes catalyze reactions that are central to the metabolic processes that sustain life. Most metabolic pathways are either net redox processes (Table 4.2) or involve redox reactions that are regulated by the redox state of the cell by transcriptional, translational, and enzyme-level control mechanisms. For example, in E. coli, the global FNR (fumarate, nitrate reduction) transcriptional regulator or the cellular redox state regulates nearly 300 genes in over 180 operons, including nearly all metabolic pathways.

Redox reactions can involve the transfer of one or two electrons during the transformation. The overall enzymatic reaction can involve a few elementary steps like glutathione reductase with five component steps. Alternatively, the reaction may be much more complex as in the pyruvate:ferredoxin oxidoreductase reaction, which involves at
least 15 steps, including binding of two substrates (pyruvate and CoA), the formation of a series of pyruvate-derived intermediates including a hydroxyethylthiamine pyrophosphate radical, multiple steps of electron transfer through the protein’s 3 [4Fe-4S] clusters, and the release of 2 products (carbon dioxide, acetyl-CoA).

Many enzymes undergo internal redox reactions. For example, the isomerase class of adenosylcobalamin-dependent enzymes catalyze 1,2 rearrangement reactions (Figure 4.18). In these reactions, the cobalt-carbon bond of deoxyadenosylcobalamin is cleaved to form a cobalt- and a carbon-centered adenosyl radical that (i) abstracts a hydrogen atom from the substrate to generate a substrate radical and deoxyadenosine. (ii) The substrate radical then rearranges to form a product radical that (iii) reabstracts the hydrogen atom from adenosine to yield the product and reform the deoxyadenosyl radical (iv). Finally, the deoxyadenosyl radical recombines with cobalt to regenerate deoxyadenosylcobalamin (v).

A number of enzymes undergo redox activation. Some enzymes require oxidative activation. For example, some enzymes like protein kinase Cγ require two specific cysteine residues to be in a disulfide state before catalysis can progress. Other enzymes require reductive activation. For example, a common theme among metal containing hydroxylases, oxidases, and oxygenases is the requirement for reduction of a metal ion (Fe$^{3+}$, Cu$^{2+}$) to its low-valent (Fe$^{2+}$, Cu$^{1+}$) state before oxygen can bind. Accordingly, cobalamin-dependent methyltransferases, which do not catalyze overall redox reactions, can suffer inactivation to the Co$^{2+}$ state under oxidative conditions. Thus, reductive activation of the cobalt ion is required before these enzymes can undergo methylation via a nucleophilic substitution reaction (Figure 4.19). In some methyl transferases, a redox
system directly reduces the $\text{Co}^{2+}$-enzyme to the active $\text{Co}^{1+}$ state (Figure 4.19A). In the case of methionine synthase (Figure 4.19B), reduction is coupled to activation and involves a reductive methylation in which S-adenosylmethionine reacts with the $\text{Co}^{2+}$ form of the cofactor to generate the methyl-$\text{Co}^{3+}$ state of the enzyme.

The enzymes (Table 4.1) and pathways (Table 4.2) that catalyze redox processes or require redox activation are being studied at different levels. Many laboratories are attempting to develop an enhanced understanding of the details of the catalytic cycles of redox enzymes and experimental approaches for uncovering such information are described in Chapter 6. Some laboratories are attempting to understand how redox enzymes and pathways are regulated at the transcriptional, translational, and enzyme level. Many of these regulatory systems themselves are subject to redox control as described in Chapter 4.3. Redox enzymology is a vast field that comprises enzymes with major significance to life’s processes.

Selected References


Table 4.1. Examples of Redox Enzymes

<table>
<thead>
<tr>
<th>Redox Enzyme</th>
<th>Redox Cofactor</th>
<th>Redox Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co Dehydrogenase</td>
<td>NiFe$_4$S$_4$, Fe$_4$S$_4$</td>
<td>H$_2$ + CO$_2$ / CO + H$_2$O</td>
</tr>
<tr>
<td>Methyl-Com Reductase</td>
<td>Ni-Coenzyme F$_{430}$</td>
<td>CH$_3$-SR + RSH / CH$_4$ + RSSR</td>
</tr>
<tr>
<td>Hydrogenase (2 Types)</td>
<td>NiFe [4Fe-4S]; [FeFe], Fe$_4$S$_4$</td>
<td>2 H$^+$ / H$_2$</td>
</tr>
<tr>
<td>Methane Monooxygenase</td>
<td>Diiron carboxylate</td>
<td>CH$_3$OH + H$_2$O / CH$_4$ + O$_2$</td>
</tr>
<tr>
<td>Superoxide Dismutase (4 Types)</td>
<td>Fe; CuZn; Ni; Mn</td>
<td>2 O$_2^-$ + 2H$^+$ / O$_2$ + H$_2$O$_2$</td>
</tr>
<tr>
<td>Heme Oxygenase</td>
<td>Heme</td>
<td>2 H$_2$ + Heme + 3 O$_2$ / biliverdin + CO + Fe$^{3+}$</td>
</tr>
<tr>
<td>Cytochrome P$_{450}$</td>
<td>Heme</td>
<td>RH + O$_2$ + H$_2$ / ROH + H$_2$O</td>
</tr>
<tr>
<td>Tyrosine Hydroxylase</td>
<td>Cu</td>
<td>RH + O$_2$ + H$_2$ / ROH + H$_2$O</td>
</tr>
<tr>
<td>Cytochrome Oxidase</td>
<td>Cu, heme, binuclear Cu</td>
<td>O$_2$ + 2 H$_2$ / 2 H$_2$O</td>
</tr>
<tr>
<td>O$_2$ Evolving Center</td>
<td>Mn$_4$CaO$_4$ cluster</td>
<td>O$_2$ + 2 H$_2$ / 2 H$_2$O</td>
</tr>
<tr>
<td>Succinate Dehydrogenase, Fumarate Reductase</td>
<td>Flavin, Fe$_4$S$_4$</td>
<td>Fumarate + H$_2$ / Succinate</td>
</tr>
<tr>
<td>Malate Dehydrogenase</td>
<td>NAD(P)$^+$</td>
<td>Oxaloacetate + H$_2$ / malate + H$_2$O</td>
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<tr>
<td>Methanol Dehydrogenase</td>
<td>Pyrroloquinoline quinone</td>
<td>HCHO + H$_2$ / CH$_3$OH</td>
</tr>
<tr>
<td>Copper Amine Oxidase</td>
<td>2,4,5-trihydroxyphenylalanine quinone</td>
<td>RCHO + NH$_3$ + H$_2$ / RCH$_2$NH$_2$ + H$_2$O</td>
</tr>
<tr>
<td></td>
<td>lysine tyrosylquinone</td>
<td>(RCH$_2$NH$_2$ + H$_2$O + O$_2$ $\rightarrow$ RCHO + NH$_3$ + H$_2$O$_2$)</td>
</tr>
<tr>
<td>Bacterial Amine Dehydrogenase</td>
<td>tryptophan tryptophylquinone</td>
<td>RCHO + NH$_3$ + H$_2$ / RCH$_2$NH$_2$ + H$_2$O</td>
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<td></td>
<td>cysteine tryptophylquinone</td>
<td>(RCH$_2$NH$_2$ + H$_2$O + Acceptor $\rightarrow$ RCHO + NH$<em>3$ + Acceptor$</em>{\text{red}}$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H$<em>2$ = reduced amicyanin, Acceptor$</em>{\text{red}}$</td>
</tr>
<tr>
<td>Xanthine Dehydrogenase</td>
<td>Mo-Pterin, Fe$_2$S$_2$, FAD</td>
<td>XH + H$_2$O + NAD$^+$ / X=O + NADH</td>
</tr>
<tr>
<td>Pyruvate Dehydrogenase</td>
<td>Thiamine pyrophosphate (TPP),</td>
<td></td>
</tr>
<tr>
<td>Enzyme</td>
<td>Co-factors</td>
<td>Reaction</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>----------------------------------------------------------------------------</td>
<td>---------------------------------------------------------</td>
</tr>
<tr>
<td>Pyruvate:Ferredoxin Oxidoreductase</td>
<td>Flavin, CoA,, lipoic acid, TPP, 3 [4Fe-4S] clusters</td>
<td>Pyruvate + CoA + H₂ / acetyl-CoA + CO₂</td>
</tr>
<tr>
<td>Ribonucleotide Reductase</td>
<td>Adenosylcobalamin, Diferric tyrosyl radical, [4Fe-4S] + glycyl radical</td>
<td>ROH + 2RSH / RH + RSSR</td>
</tr>
<tr>
<td>Glutathione Reductase</td>
<td>FAD, RSSR / (RSH)₂ (R = Cys)</td>
<td>Glutathione disulfide / 2 GSH</td>
</tr>
</tbody>
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Table 4.2. Examples of Redox Pathways:

<table>
<thead>
<tr>
<th>Photosynthesis (O&lt;sub&gt;2&lt;/sub&gt; evolution)</th>
<th>Methanogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiration</td>
<td>Acetogenesis</td>
</tr>
<tr>
<td>Oxidation of Fats, Sugars, Nucleic acids</td>
<td>Sulfate reduction</td>
</tr>
<tr>
<td>Fermentations (glycolysis, fatty acid oxidation, nucleotide oxidation)</td>
<td>Sulfur oxidation</td>
</tr>
<tr>
<td></td>
<td>Metal oxidation and metal reduction</td>
</tr>
</tbody>
</table>
**Figure 4.18** Internal redox reactions catalyzed by adenosylcobalamin-dependent isomerasers.
Figure 4.19  Reductive activation is required for cobalamin-dependent methyltransferases. X designates a variety of methyl donors like hydroxide, tetrahydrofolate, thiolates, and amines and Y specifies various methyl acceptors like homocysteine and coenzyme M.
4.7 Circadian Clock and Heme Biosynthesis

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In response to daily environmental cues, endogenous circadian oscillators control the behavior of many living organisms. The endogenous clock is characterized by a self-sustaining cycle of approximately 24 hours in duration that acts as a pacemaker for cellular and behavioral processes such as the sleep-wake rhythm. Fundamentally, the circadian clock regulates a range of physiological and biochemical processes necessary to maintain metabolic homeostasis. How the circadian clock accomplishes this task is of great biological interest.

4.7.A Cyclic expression of heme binding proteins

It has been observed that activities of many hemoproteins such as nitric oxide synthase, guanylyl cyclase, catalase, prostaglandin synthase, cytochrome oxidases/P450 display diurnal pattern. Gene expression studies suggest that the transcription of these genes is under circadian regulation in vivo. These hemoproteins are often the rate-limiting regulators of their respective biochemical cascades. Essential for their biochemical function is the prosthetic moiety heme. Heme is necessary for electron flow, as in cytochrome-mediated electron transport in mitochondria and for sensing gaseous signals as in guanylate cyclase. At the cellular level, these observations raise the question of how temporal production of these rate-limiting biological regulators and the heme prosthetic moiety are coordinated.

Heme biosynthesis starts and ends in the mitochondrion and requires multiple enzymatic steps. The first enzyme in this pathway is aminolevulinate synthase 1, or unique to erythrocytes, aminolevulinate synthase 2, which catalyzes condensation of glycine and succinyl-CoA into aminolevulinic acid. Cells incubated with varying glycine and succinyl CoA concentrations do not produce proportional levels of heme in contrast to those treated with aminolevulinate acid, indicating that aminolevulinate synthase 1 is the rate-limiting step. Free heme is known to negatively regulate aminolevulinate
synthase 1 expression but the exact feedback mechanism remains unclear. Gene expression analysis revealed that both aminolevulinate synthase 1 and aminolevulinate synthase 2 display a robust 24 hour temporal profile indicating that they are under circadian control in vivo (Figure 4.20). This conclusion is consistent with the observation that the diurnal pattern of aminolevulinate synthase 1 gene expression is deregulated in circadian deficient genetically altered mice. Other studies also demonstrate circadian control of aminolevulinate synthase expression in fruit flies, indicating that the temporal regulation is evolutionarily very ancient. To illustrate the relationship between heme biosynthesis and circadian control, an understanding of the current circadian clock mechanism is necessary.

4.7.B Circadian clock mechanism

Our current knowledge of the mammalian clockwork has been aided in part by genetic studies with the fruit fly, Drosophila melanogaster. The fruit fly clock is based on a transcription/translation autoregulatory mechanism. Mammalian homologs to the Drosophila circadian regulators include the bHLH PAS transcription factors CLOCK and BMAL. The mammalian CLOCK has a paralog known as NPAS2. CLOCK or NPAS2 will heterodimerize with BMAL1 to form the core transcriptional complex whose activity is modulated by feedback regulators such as cryptochrome (mCRY1 and mCRY2), and Period (mPER1 and mPER2). Other modulators of the circadian mechanism include casein kinases (epsilon and delta) that regulate phosphorylation of circadian regulators that in turn control its stability and degradation. In addition, the orphan nuclear receptors Rev-Erbα and RORα regulate transcription of Bmal1 gene that in turn controls the availability of BMAL1 level. Mutations in genes encoding these regulators in the mouse affect, to varying degrees, the temporal expression of cellular functions and animal behavior such as sleep-wake rhythm. The loss of function mutations of Bmal1 and mPer2 severely impact the mouse circadian clock rhythm. One view is that mPER2 may be a rate-limiting modulator of the mammalian circadian clock mechanism. Mice carrying double mutations of either mCry1/mCry2 or mPer1/mPer2 display no circadian rhythm in sleep-wake behavior. While there are similarities in function between the circadian
homologs in fruit fly and mouse there are also significant differences in their respective roles in the clock mechanism. For example, it remains unclear whether the mouse *Timeless* homolog is a circadian regulator. In addition, multiple mammalian homologs of the respective fruit fly gene were observed. In fruit fly and zebrafish, cryptochromes serve as the circadian photosensor, but mouse cryptochromes have apparently lost this photoreception function. Instead, the circadian photoreception is regulated in part by melanopsin, a member of the opsin photosensor family. The *cryptochromes* are FAD binding proteins belonging to the plant blue light receptor family. They have peptide sequence homology to DNA photolyase but have no DNA repair function. That mCRY plays a negative modulating role on the core transcriptional complex activity is supported by key observations. Firstly, expression of *mPer1* is elevated in genetically modified mice deficient in cryptochromes. Secondly, reporter assay studies demonstrate mCRYs are potent inhibitors of the BMAL1/CLOCK or BMAL1/NPAS2 circadian transcription complex activity. While early reporter assay studies suggested mPER2, like its fruit fly counterpart dPER, is a negative regulator, other studies showed that mPER2 did not significantly inhibit BMAL1/CLOCK transcription complex activity. Critically, the loss of mPER2 function in mice dampened expression of *Bmal1*, *mPer1* or *mCry1*, implicating it as a positive regulator *in vivo*. Thus, the molecular target and function of mPER2 was an enigma.

4.7.C PAS is a heme binding domain

Important insight into the role of heme in circadian clock function came from analysis of the PAS domain structure shared among circadian regulators including CLOCK, BMAL1, NPAS2, mPER1 and mPER2. In mammals, the PAS domains are two conserved 51 amino acid repeats known as PAS-A and PAS-B. The PAS domain has three-dimensional folds that are highly conserved from bacteria to mammals. The PAS domain of proteins such as AxPDEA1, DOS, FIX and NPAS2 is the heme binding motif. PAS proteins such as FIXL bind one heme molecule whereas NPAS2 binds two heme molecules, one each to the PAS-A and PAS-B. *In vitro* studies with reconstituted peptides have demonstrated that NPAS2/BMAL1 does not bind DNA in the presence of
carbon monoxide and heme. However, carbon monoxide or heme alone does not affect the ability of NPAS2/BMAL1 to bind DNA. Thus, these in vitro studies demonstrate that heme could control BMAL1/NPAS2 transcription complex activity by DNA binding inhibition in response to carbon monoxide.

The effects of free heme on the expression of key clock regulators further illustrate its importance in the circadian clock mechanism in vivo. Heme injected into mice enhanced mPer1 but dampened mPer2 expression. In contrast, expression of Bmal1, Npas2, mCry1 and Clock were apparently unaffected in vivo. Since the BMAL1/NPAS2 transcription complex regulates expression of mPer1 and mPer2, the findings that heme modulates the circadian transcriptional activity is consistent with the in vitro observations.

4.7.D Expression of Npas2 is controlled by mPER2

A finding that revealed key insight into the circadian mechanism was that the loss of mPER2 function resulted in the loss of Npas2 but not Clock expression in vivo. The observation suggests that the molecular target of mPER2 is NPAS2 rather than CLOCK. Reporter assay studies demonstrate mPER2 positively activates BMAL1/NPAS2 transcription complex activity but not that of BMAL1/CLOCK. The finding would also be consistent with observations that implicate mPER2 as a positive regulator of the clock transcription mechanism in vivo. Together, these findings indicate that mPER2 is a major regulator of NPAS2 and not CLOCK in vivo. Recent studies have revealed that mice carrying a loss of function mutation of CLOCK displayed circadian behavior similar to wild type animals. It raises the possibility that the phenotype of the original Clock mutant mice was a gain of function. Thus, the exact role played by CLOCK in the mammalian circadian mechanism remains unclear.

4.7.E NPAS2 regulates expression of aminolevulinate synthase 1

Given that aminolevulinate synthase 1 expression is under circadian control, it raises the question whether NPAS2 is a key regulator. Mice deficient in NPAS2 function displays reduced expression of aminolevulinate synthase 1 confirming that NPAS2 is a major transcriptional regulator of its expression. Therefore, BMAL1/NPAS2 transcription
complex control of aminolevulinate synthase 1 expression closes the loop on a reciprocal regulation between circadian clock regulation of heme biosynthesis and heme control of circadian clock complex regulated transcription. A model (Figure 4.21) illustrates the expression of aminolevulinate synthase 1 is dependent on BMAL1/NPAS2 transcription activity under positive regulation by the mPER2 protein. Via biosynthesis, the level of heme is controlled by the level of aminolevulinate synthase 1 expressed. Heme then binds to NPAS2 and mPER2 proteins via the PAS domains to activate their function. In addition, heme biosynthesis is coordinated with the expression of circadian controlled genes that encode hemoproteins such as nitric oxide synthase, catalase, guanylyl cyclase and others that are key regulators of various biochemical and physiological cascades.

Increased levels of heme eventually induce its own degradation via heme-oxygenase enzymes whose gene expression is induced by free heme. Degradation of heme by heme-oxygenases results in the formation of carbon monoxide, biliverdin and free iron. In the presence of carbon monoxide, heme bound to the BMAL1/NPAS2 transcription complex stops DNA binding and by extension, gene transcription. The decline in transcription complex activity is further amplified by a decreased level of mPER2. The consequence is lower aminolevulinate synthase 1 expression leading to a decreased heme level. The released transcription complex components are then presumably targets for degradation. The decline in heme level eventually reaches a trough that in turn allows a basal level BMAL1/NPAS2 transcription complex to bind DNA, thus restarting the feedback cycle. Additional evidence of heme role in regulating the circadian mechanism has come from independent studies. The transcription of Bmal1 gene is regulated by the orphan nuclear receptors Rev-Erbα. Mutation of Rev-Erbα in mouse or its Drosophila homolog E75 affects circadian behavior. Recent studies demonstrate that E75 is a heme binding protein. Thus, the role of heme in the circadian mechanism is likely to be broad. In conclusion, the reciprocal regulation between heme biosynthesis and the circadian clock mechanism allows output signals from the cellular/subcellular environment to provide feedback regulation on the core clock mechanism.

Selected References


Figure 4.20  Circadian expression of mouse aminolevulinate synthase 1 (mAlas) gene during a daily cycle. The analysis of gene expression was carried out by Northern blotting of liver mRNA obtained from mouse livers at the indicated time points with radiolabeled cDNA probes. A duplicated set of experiment is shown to illustrate the lack of temporal regulation of Alas1 expression in circadian deficient animals carrying mutation of mPer1 and mPer2 genes. Levels of glycoaldehyde 3-phosphate dehydrogenase (Gapdh) mRNA were used as an internal control. The lower panel shows the quantification of the band intensity of the Northern blot with respect to the mRNA of Gapdh.
**Figure 4.21** A model for reciprocal regulation between the circadian clock and heme biosynthesis. Mammalian Period protein 2 (mPER2), Neuronal PAS domain protein 2 (NPAS2), Brain-Muscle-ARNT-Like protein 1 (BMAL1), Heme-Oxygenase (HO), Aminolevulinate Synthase 1 (ALAS1), Carbon Monoxide (CO), and Heme (4 red squares with Fe).