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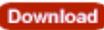
## From redox homeostasis to protein structure modulation and redox signaling therapy

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**Reactive oxygen species (ROS) have been identified as being responsible for many harmful events in humans, while on the other hand, they are increasingly being recognized as necessary cell signaling agents. The multiple roles of ROS are reflected in the multiple roles of antioxidants, which are part of many disease preventing remedies, but are recently also discussed as disease causing agents. This article provides an overview of the current knowledge of ROS as part of complex redox networks that can harm or benefit cells. It presents up-to-date physicochemical and biological information, in an understandable, novel way, to involve the reader in the significant discussion on ROS and anti-ROS as the novel therapeutic agents of the coming decades. Section I of the article introduces the chemical nature of redox reactions and how their redox potentials determine a biological redox set point that is controlled within narrow margins. Section II presents how a healthy cell continually senses and regulates the redox set point as a key feature of life, how reactive oxygen species as key factors disturb the homeostatic redox set point, and how antioxidants as key compensatory factors act to counterbalance the disturbances. Section III introduces the notion that redox regulation involves intentional subtle changes of the redox set point for normal cell behavior and how this regulative feature of ROS makes them good candidates for novel therapies. After reading this article, the reader will understand chemical terms such as half reaction and standard redox potential, biological terms such as singlet oxygen and ROS signaling, and therapeutic terms such as small molecule therapy and signal transduction modulators. These and other terms are necessary to understand for the informed discussion of novel human therapies based on redox homeostasis.**

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# From Redox Homeostasis to Redox Signaling Therapy

Prepared for ENG3 Corporation, Seattle

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## From Redox Homeostasis to Redox Signaling Therapy

Redox signaling in biological systems is a highly complex, not yet well understood, process of sensing and regulating a cell's electrochemical state. Related terms often used to refer to this property of a cell include electrochemical potential, redox tone, and redox homeostasis. The main objective of this review is to present information on how a cell's homeostatic, or normal, steady redox-state is maintained and regulated. Special emphasis is given to the role of reactive oxygen species (ROS) and other small signaling molecules whose biological role is modulated by ROS. At the outset we include a short review of basic redox chemistry and its significance to regulating protein structure and function. We conclude with a discussion of recent redox discoveries that may lead to novel human therapies based on redox signaling.

### I Redox Homeostasis and the Redox Set Point E'

The term *redox* is an acronym for “reduction and oxidation” and refers to the class of chemical reactions that proceed with the gain (chemical reduction) or loss (chemical oxidation) of one or more electrons; or to the electrochemical equilibrium state of such reactions. The blending of the two words into one new term is justified since reductions and oxidations are linked as illustrated in reaction 1 (R 1). One compound,  $A_{\text{reduced}}$ , becomes oxidized by giving up electrons and a second compound,  $B_{\text{oxidized}}$ , becomes reduced by acquiring the electrons lost by  $A_{\text{reduced}}$ .



R 1

For every oxidation reaction that occurs, a concurrent reduction reaction occurs and vice versa. Compensatory chemical changes in A and B occur to accommodate the actual electron transfer reactions as shown in Figure 1.

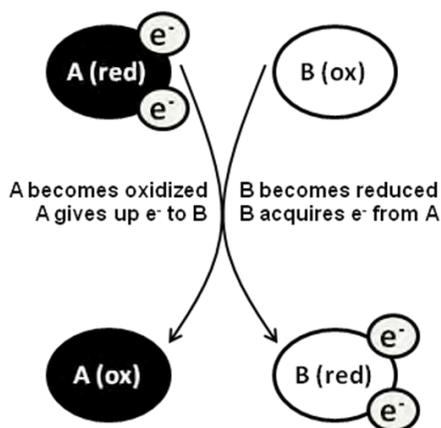


Fig. 1: Redox reactions including a transfer of electrons from a reduced compound, A(red), to an oxidized compound, B(ox). In the process, A becomes oxidized and B becomes reduced.

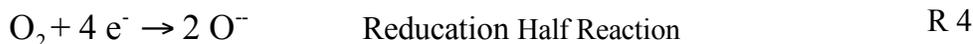
Redox reactions are at work all around us. For example, the prominent darkening of the surface of metallic copper (Cu) cookware during heating is caused by the heat-accelerated oxidation of

the Cu atoms to the oxidized form known as copper oxide (CuO), while at the same time atmospheric oxygen is reduced as shown in R 2.



R 2

Redox reactions, like that shown in R 2, are most easily understood when rewritten as a combination of two *half reactions*. One half reaction is called the oxidation half reaction, while the other half reaction is known as the reduction half reaction. For the case of the oxidation of Cu by O<sub>2</sub> the two half reactions are shown in R 3 and R 4, with R 5 representing the sum of the two half reactions, or the overall reaction.



The chemical species that constitute an oxidation half reaction (e.g., Cu and Cu<sup>++</sup> in R 3) are also known as an *oxidation couple* while the corresponding pair of chemicals involved in the reduction half reaction (e.g., O<sub>2</sub> and O<sup>-</sup> in R 4) are known as the *reduction couple*. Together, two such couples are often spoken of as a *conjugate pair* (e.g. Cu and O<sub>2</sub> in R 5). In our example, O<sub>2</sub> is called the *oxidizing agent* of R 5, the substance that gains electrons, and Cu acts as the *reducing agent*, the substance that provides electrons for oxygen.

In biological systems, which include both the intracellular and extracellular environments, there are an abundance of redox couples and conjugate pairs. As one example, figure 2 shows the redox couples of H<sub>2</sub>/H<sup>+</sup> and O<sub>2</sub>/O<sup>-</sup> and the conjugate pair of H<sub>2</sub> and O<sub>2</sub> in analogy to the Cu/CuO system discussed above.

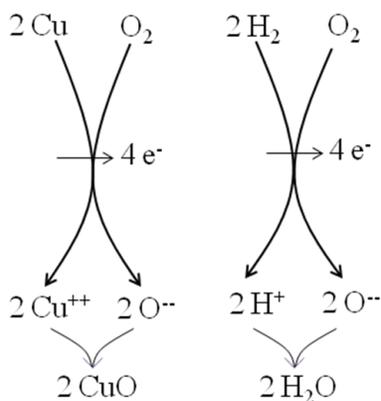
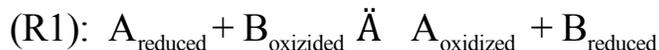


Fig. 2: The redox couples Cu/Cu<sup>++</sup>, O<sub>2</sub>/O<sup>-</sup>, and H<sub>2</sub>/H<sup>+</sup> and the conjugate pairs Cu/O<sub>2</sub> and H<sub>2</sub>/O<sub>2</sub>. The directions of the individual reactions, as illustrated by the arrow heads, depend on the relative concentrations of the individual components and their electrochemical characteristic, as explained in more detail in the text.

All cellular redox couples are in a complex, dynamic, physicochemical equilibrium and their quantitative interactions determine a cell's steady state *redox set point*. Under normal conditions this set point is maintained in a relatively constant state that is often referred to as the *homeostatic redox state* of the cell. To appreciate the contribution that individual redox couples make to a cell's integrated homeostatic redox state, it helps to understand the electrochemical

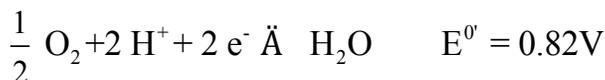
forces that define the course of these reactions, but ultimately these electrochemical forces must be considered in conjunction with biological controls to adequately understand how biological redox set points are achieved.

Reaction 1 is written as an *equilibrium reaction*.



This is defined as a reaction state, in which there is no net change in concentration of the constituents of the reaction, but in which there is a continuous conversion of reactants ( $A_{\text{reduced}}$  and  $B_{\text{oxidized}}$ ) to products ( $A_{\text{oxidized}}$  and  $B_{\text{reduced}}$ ) and an exactly balancing conversions of products to reactants. All other things being equal, if a quantity of one reactant, such as  $A_{\text{reduced}}$  is added to such an existing equilibrium state mixture of all reactants and products, then additional quantities of products will appear while the reactants will decrease in concentration until a new equilibrium state is attained. The effect of the addition of  $A_{\text{reduced}}$  to the mixture is an example of the simple principle of *Mass Action* which states that changes in the concentration of individual reaction components can drive the reaction to the right or left, depending on which component is added (or subtracted).

The electrochemical or redox reactions that concern us in this discussion have an addition fillip that tends to complicate the simple concept of mass action. Each individual redox couple is characterized by its unique tendency to gain or lose electrons and this tendency is known as the couple's *Standard Redox Potential*. This potential is almost universally presented in tabulations as the reduction form of a half reaction (in which electrons are gained) and is then known as the *Standard Reduction Potential* symbolized as  $E^{\circ}$  for biological half reactions. Reaction 6 shows the reduction half reaction of molecular oxygen with hydrogen and its related biological standard reduction potential.



R 6

The numerical value of  $E^{\circ}$  is determined experimentally by measuring the voltage generated between any given reaction couple and a hydrogen/hydrogen ion couple. The reduction of hydrogen ion to hydrogen gas is the reference point which is considered to have a voltage of -0.421 volts. To establish  $E^{\circ}$  the chemical and physical conditions under which the voltage measurement is made are rigidly defined. It is required that the voltage is measured when all solutes are present at a concentration of 1 Molar, gases are at a partial pressure of 1 atmosphere, the pH is 7.0 ( $10^{-7}$  Molar  $H^+$  concentration), and the temperature is  $25^{\circ}C$ . Figure 3 shows an arrangement called a Galvanic cell, which is used to measure  $E^{\circ}$ . It consists of two half-cells, one for the hydrogen/hydrogen ion couple, the other for the reaction couple to be measured.

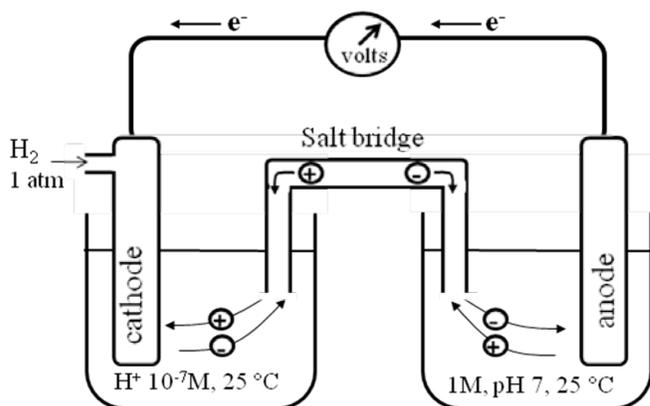


Fig. 3: Galvanic cell to measure the standard reduction potential of a redox pair that is present in the right half cell. Its tendency to be reduced is compared to the tendency of  $\text{H}_2/\text{H}^+$ , present in the left half cell.

The standard reduction potential specifies the relative tendency of a substance to gain or lose electrons compared to the standard hydrogen half cell. A positive voltage such as for  $\text{O}_2$  in R 6 indicates a strong tendency to accept electrons, and to oxidize chemicals whose standard reduction potential is less positive. Hence, oxygen is a strong oxidant or oxidizing agent, which in the presence of hydrogen has the tendency to become water. For comparison,  $E^{0'}$  of the  $\text{NADP}^+/\text{NADPH}$  couple, as shown in table 1, is -0.32 volts, which indicates that this couple will transfer electrons to oxygen under standard conditions. The biological importance will become evident below when nicotinamide adenine dinucleotides/phosphates ( $\text{NAD}^+/\text{NADP}^+$ ) are introduced as important reducing agents that are involved in the protection of cells against damaging reactive oxygen species.

With this background, we can now address the forces that influence reaction R 1.



When we add a quantity of  $A_{\text{reduced}}$  to an existing equilibrium state mixture, the driving force pushing the reaction to the right is the sum of the change in concentration of  $A_{\text{reduced}}$  plus the added electron donating potential of the added  $A_{\text{reduced}}$ . This concept of a dual driving force for electrochemical reactions is quantified by the *Nernst equation*.

The Nernst equation for the half reaction in which  $A_{\text{oxidized}}$  is converted to  $A_{\text{reduced}}$  is shown in equation 1 (Eq 1).

$$E = E^{0'} - \frac{RT}{zF} \ln \frac{A_{\text{(reduced)}}}{A_{\text{(oxidized)}}}$$

Eq 1

$E$  represents the total driving force for the reaction under any existing set of concentrations of  $A_{\text{oxidized}}$  and  $A_{\text{reduced}}$  and is called the *electromotive force*.  $E^{0'}$  is the standard reduction potential for the half cell;  $z$  is the stoichiometric number of electrons transferred per reaction;  $R$ ,  $T$ , and  $F$  are the gas constant, the absolute temperature, and the Faraday constant, respectively. Hence, the Nernst equation can be used to calculate the potential of a half cell under non standard conditions

such as present in a biological cell. As an aside, it can be shown that under standard conditions the logarithmic term becomes zero and then  $E = E^0$

Table 1 shows the reduction half reactions and the standard electrode reduction potentials  $E^0$ , of a few well studied redox couples that operate in biological systems and that are relevant to our discussion in this article. The compounds in the upper part of the table are listed with the most strongly reducing redox couple at the top and the most strongly oxidizing couple at the bottom. Electrons flow spontaneously from the more readily oxidized redox couple (the one with the more negative  $E^0$ ) to the more readily reduced redox couple (the one with the more positive  $E^0$ ). Below the list of redox couples, two half reactions of hydrogen peroxide are given to show that some agents can function as either oxidizing or reducing agent, dependent on the conjugate partner. Finally, table 1 shows the pH dependency of  $E$  using oxygen as example. In general,  $E$  becomes more positive, i.e. the oxidizing capacity increases, as the pH decreases. As useful as standard potentials may be in predicting the tendency of redox substances to react in a self-sustaining way, they cannot predict the ease and the speed (i.e. the kinetics) with which the reactions happen.

Selected Redox Couples	Reduction Half Reactions	Standard Electrode Potentials
Nicotinamide adenine dinucleotide phosphate	$\text{NADP}^+ + 2 e^- \rightleftharpoons \text{NADPH} + \text{H}^+$	-0.32V
Thioredoxin	$\text{TrxSS} + 2 e^- + 2 \text{H}^+ \rightleftharpoons \text{Trx(SH)}_2$	-0.28 V*
Glutathione	$\text{GSSG} + 2 \text{H}^+ + 2 e^- \rightleftharpoons 2 \text{GSH}$	-0.25 V*
Cysteine	$\text{CysS-SCys} + 2 \text{H}^+ + 2 e^- \rightleftharpoons 2 \text{CysSH}$	-0.15 V*
Hydrogen	$2 \text{H}^+ + 2e^- \rightleftharpoons \text{H}_2$	-0.42 V
Copper containing compounds	$\text{Cu}^{2+} + 2 e^- \rightleftharpoons \text{Cu}$	+0.34 V
Iron containing compounds	$\text{Fe}^{3+} + e^- \rightleftharpoons \text{Fe}^{2+}$	+0.77 V
Hydrogen Peroxide	$\text{O}_2 + 2 \text{H}^+ + 2 e^- \rightleftharpoons \text{H}_2\text{O}_2$	+0.68 V
	$\text{H}_2\text{O}_2 + 2 \text{H}^+ + 2e^- \rightleftharpoons 2 \text{H}_2\text{O}$	+1.78 V
Oxygen (pH 14)	$\text{O}_2 + 4 e^- + 2 \text{H}_2\text{O} \rightleftharpoons 4 \text{OH}^-$	+0.40 V
Oxygen (pH 7)	$\text{O}_2 + 4 \text{H}^+ + 4 e^- \rightleftharpoons 2 \text{H}_2\text{O}$	+0.82 V
Oxygen (pH 0)	$\text{O}_2 + 4 e^- + 4 \text{H}^+ \rightleftharpoons 2 \text{H}_2\text{O}$	+1.23 V

\* Jones et al., 2004

To cap this brief review of basic redox homeostasis we can draw a conceptual analogy between batteries and biological redox set points. Just as batteries have positive and negative poles and voltage values that reflect their composition and state of charge, biological redox reactions also have positive and negative voltages whose value depends on the composition and concentration of the components of the couple. If a heterogeneous set of batteries are connected in parallel they will equilibrate and display a single voltage, or set point, that depends on the conditions and composition of that specific set of batteries. Likewise in a cell, or other biological system, the redox set point is represented by the integrated value of the  $E^{0'}$  values of all the system's redox reactions and the concentration of all chemical species involved in those reactions. To actually arrive at a calculation of a biological redox set point (or the value for the redox homeostatic state) is beyond the scope of this article.

In summary, there are a large number of redox couples in a biological system. These are all in a steady state equilibration with each other, and this equilibrated state is the system's redox set point. The set point is an important factor for the viability of a biological system. The next section will identify reactive oxygen species as key factors that disturb the homeostatic biological redox set point and present antioxidants as the key compensatory factors that act to counterbalance the disturbances.

## II Biological Relevance of the Redox Set Point $E'$

### 2.1 Relevance of Reactive Oxygen Species for $E'$

The redox set point that is observed in normal cells is that of a relatively reducing environment. While it is difficult to derive the exact overall cellular reduction potential, the redox state of one particular redox couple, glutathione/ glutathione disulfide (GSH/GSSG), is sometimes used as a first approximation. The reason for this is that GSH/GSSG is quantitatively the major intracellular redox component. Glutathione's role in the cell is discussed in more detail below in "Major Cellular Redox Couples."  $E^{0'}$ <sub>GSH/GSSG</sub> lies between -2.40 V and -1.70 V, dependent on the biological activity of the cell (Schafer and Buettner, 2001). However, these numbers can be substantially different in different cellular compartments (Smith, 2005). In any case, changes to the homeostatic set point (or the set points) can have profound consequences to cell structure and function. The main perturbations on normal redox homeostasis are generated by molecular oxygen and the reactive molecules that are formed as intermediate products when inhaled oxygen is metabolized stepwise to water (Apel and Hirt, 2004). These intermediate molecules are collectively known as *Reactive Oxygen Species (ROS)* and the cellular redox disturbances caused by ROS are called *oxidative stress*.

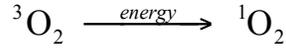
ROS cause oxidative cell stress because they are ubiquitously formed and they are highly reactive as a consequence of the fact that they are almost all *free radicals* (Figure 4).

Radical Name	Symbol	Lewis Structure	Half-Life	Orbitals	
				2p $\pi^*$	2p $\sigma^*$
Hydroxyl	$\cdot\text{OH}$		Nanosecond	$\uparrow\downarrow\uparrow\downarrow$	$\uparrow$
Superoxide	$\cdot\text{O}_2^-$		Microsecond	$\uparrow\downarrow$	$\uparrow$
Nitric oxide	$\cdot\text{NO}$		Seconds	$\uparrow$	
Triplet oxygen	$^3\text{O}_2$		Stable	$\uparrow$	$\uparrow$
For comparison:					
Water	$\text{H}_2\text{O}$		Stable	$\uparrow\downarrow\uparrow\downarrow$	$\uparrow\downarrow$

Fig. 4: Examples of free radicals including molecules and ions with unpaired electrons. These are represented in the radical symbol by a dot before the structure, in the Lewis structure by an isolated single electron dot, and in the electron spin diagram by a single arrow in one or more of the outer orbitals. Free radicals are typically highly reactive and short lived with the exception of triplet oxygen.

Free radicals are unstable molecules that form as a consequence of special, *one-electron redox reactions* that cause molecules to gain or lose unpaired electrons. In stable molecules, such as  $\text{H}_2\text{O}$  in Figure 4, electrons exist in pairs in which the individual electrons of the pair spin in opposite directions in orbitals at a thermodynamically defined distinct distance from the atomic nucleus. These opposing spin states are expressed as  $+\frac{1}{2}$  and  $-\frac{1}{2}$  or as up- and downward arrows ( $\uparrow\downarrow$ ). In various publications the unpaired electron of a free radical is symbolized by a dot before or after the chemical formula. In this article the convention is that free radicals are symbolized with a dot before the structure. Figure 4 also shows the electronic Lewis structures of the ROS species, in which the free radical electrons are symbolized as single dots and the electron pairs as double dots.

The examples in figure 4 include *triplet oxygen* as a free radical. This “ground state” oxygen is the dominant form of oxygen in the air around us. It is a relatively exceptional molecule in that it has two unpaired electrons and can thus be viewed as a diradical. Since the electrons are not in the same orbital, they can assume three possible arrangements ( $\uparrow\uparrow$ ,  $\downarrow\downarrow$ , and  $\uparrow\downarrow$ ), which is known as the triplet state and chemically symbolized as  $^3\text{O}_2$ . Figure 4 shows the electrons in same parallel spin state (symbolized  $\uparrow\uparrow$ ). Unlike most free radical species, triplet oxygen is not particularly reactive under normal biological conditions, and hence, is a long-lived substance. With the addition of energy, triplet oxygen is readily converted to a more reactive species known as *singlet oxygen* ( $^1\text{O}_2$ ) as shown in R 7.



R 7

Sufficient energy flips the spin state of one of the radical electrons (+ ½ to – ½, or vice versa) yielding an electron configuration where the two diradical electrons spin in the opposite direction in the same orbital. This energy activated spin state is known as the anti-parallel spin state, symbolized as ↑↓. As a consequence of its additional energy content  ${}^1\text{O}_2$  is more reactive than  ${}^3\text{O}_2$  and has a half-life in the microsecond range.

Figure 5 shows some major ROS species and how they are derived from one another. The biological significance of these ROS species is discussed in more detail in the following text.

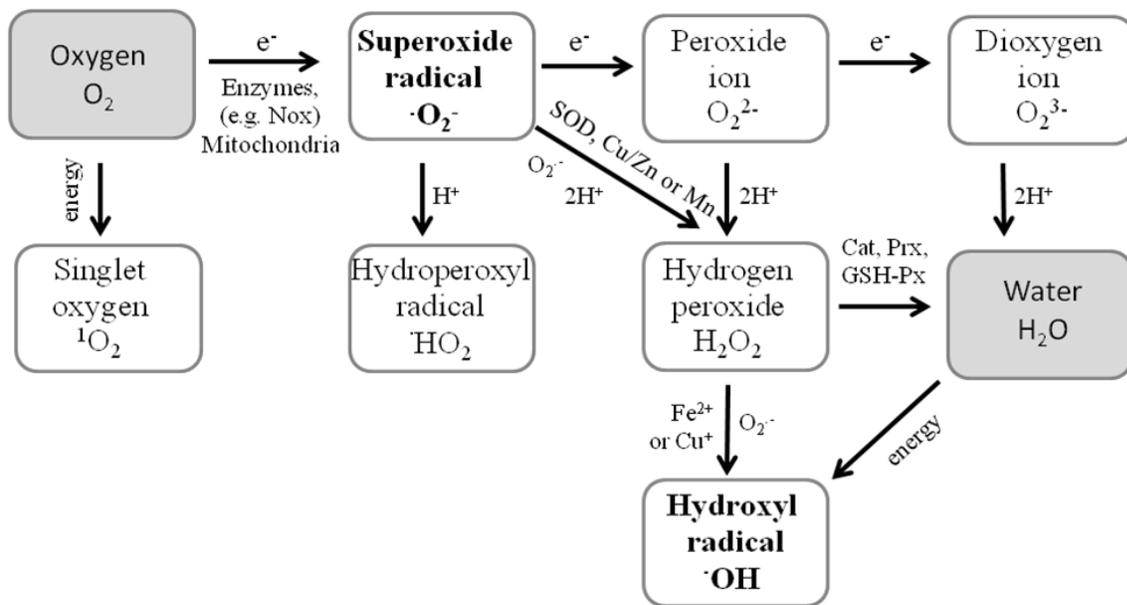
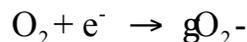


Fig. 5: Reactive oxygen species (ROS) as formed during the reduction of oxygen to water. The majority of ROS are short lived free radicals, such as superoxide and hydroxyl radical. Some molecules are not free radicals themselves, such as singlet oxygen, but catalyze the formation of free radicals. ROS further include reactive but comparatively long-lived molecules such as hydrogen peroxide. SOD: superoxide dismutase (Cu/Zn isoform in cytoplasm, Mn isoform in mitochondria), Cat: catalase, Prx: peroxiredoxin, GSH-Px: glutathione peroxidase. For more details, see text.

One-electron reduction of molecular oxygen (O<sub>2</sub>) leads to *superoxide radical* (·O<sub>2</sub><sup>-</sup>) as shown in R 8.

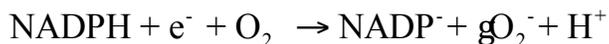


R 8

The main source of ·O<sub>2</sub><sup>-</sup> is aerobic respiration inside mitochondria, where at least nine production sites of ·O<sub>2</sub><sup>-</sup> are known (Andreyev et al. 2005). This superoxide has historically been viewed as a

toxic byproduct of normal mitochondrial respiration, formed when electrons leak from their normal reaction pathway and react with triplet oxygen.

The conversion of oxygen to superoxide is also catalyzed by several enzymes. The most ubiquitous ones are *NADPH-oxidases*, abbreviated as Nox (Behe and Segal, 2007). Nox, which are in fact enzyme complexes, are used by phagocytic white blood cells such as macrophages and neutrophils to produce millimolar quantities of  $\cdot\text{O}_2^-$  as shown in reaction 9.



R 9

In these white blood cells, the generated  $\cdot\text{O}_2^-$  is restrained inside the vacuole that is formed during phagocytosis, where it helps to degrade the ingested particles. The production of  $\cdot\text{O}_2^-$  leads to the generation of other toxic free radicals and the process in its entirety is called a *respiratory burst*. In humans, seven isoforms of the key Nox catalytic enzyme subunit have been found in non-phagocytic cells. In these cells, the production of ROS plays an important role in redox signaling as we will see in Section III of this article.

In addition to Nox, other enzymes are known to be involved in the generation of superoxide. Examples are xanthine oxidases, lipoxygenases, cyclooxygenases, and P-450 monooxygenases. These enzymes are involved in degradation of fatty acids, microsomal destruction of drugs, arginine metabolism, and other cellular actions. It is beyond the scope of this article to present their distributions and actions in detail.

The charged superoxide radical is in equilibrium with the uncharged *hydroperoxyl radical* ( $\cdot\text{HO}_2$ , figure 5). At pH 7, only one percent of the pair is in this uncharged form and, since charged molecules are not freely diffusible across biological membranes the bulk of the radicals remain trapped inside membranous organelles such as mitochondria. However, like all chemical reactions, free radical reactions are pH dependent. For instance, at pH 5, compared to the normal cellular pH of 7.0, superoxide radicals become significantly more protonated, electrically neutral, and can easily pass through membranes. Under this circumstance – which occurs for instance as part of the degradation of phagocytosed particles – the concentration of  $\cdot\text{HO}_2$  is about equal to the concentration of  $\cdot\text{O}_2^-$ , which means that at least half of the previously restrained radicals can pass into the cytoplasm.

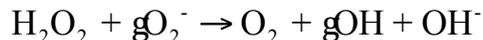
One-electron reduction of superoxide leads to the formation of *peroxide ion*  $\text{O}_2^{2-}$ , which in its protonated (neutralized) form is called *hydrogen peroxide* ( $\text{H}_2\text{O}_2$ , figure 5).  $\text{H}_2\text{O}_2$  has a relatively long lifetime (seconds to minutes) compared to other ROS, whose lifetimes are only fractions of seconds. Furthermore,  $\text{H}_2\text{O}_2$  is less reactive than other ROS and it can easily be transformed into water by catalases (cat) and peroxidases (Prx and GSH-Px). All this makes  $\text{H}_2\text{O}_2$  a good candidate for ROS signaling and consequently we will discuss the biological role of this small versatile molecule in detail in Section III under 3.1 below.

$\text{H}_2\text{O}_2$  can be very dangerous to the cell, since it can readily lose one electron and a proton to generate the highly reactive *hydroxyl free radical* ( $\cdot\text{OH}$ , figure 5), which is the free radical of the hydroxide ion  $\text{OH}^-$ . In this way,  $\text{H}_2\text{O}_2$  can act as a carrier that migrates due to its long lifetime to places, such as the nucleus, where hydroxyl free radicals would have not reached by themselves. For the generation of  $\cdot\text{OH}$  from  $\text{H}_2\text{O}_2$  to occur, oxidation of a reduced heavy metal ion such as  $\text{Fe}^{2+}$  or  $\text{Cu}^+$  is typically necessary. The reaction, also known as the *Fenton Reaction*, is shown as R 10.



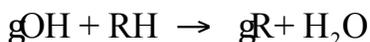
R 10

The oxidized metal,  $\text{Fe}^{3+}$  in R 10, can then further catalyze the reaction between  $\cdot\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  to produce even more hydroxyl radicals as shown in R 11.



R 11

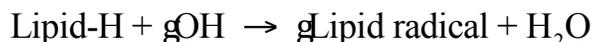
Free radicals such as hydroxyl radicals have the need to become stable again. To do so, they either steal electron(s) from other molecules, they donate their unpaired electron(s) to other molecules, or they share their outer orbital electron(s) with other molecules. In all cases, the electron deficiency of the original free radical is satisfied and the target molecules themselves become free radicals as a consequence of now having an unpaired electron. This is shown in R 12 using a hydroxyl radical as example and a biomolecule, generalized as RH.



R 12

Hydroxyl radicals have a strongly positive  $E^0$ , which makes them especially reactive compounds with a very short half-life of nanoseconds. The biomolecule RH of R 12 can be almost any type of biomolecule that is in the vicinity of  $\cdot\text{OH}$ . Thus,  $\cdot\text{OH}$  can damage a carbohydrate by randomly removing a hydrogen atom from one of its carbon atoms. It can damage DNA by altering DNA bases and by breaking hydrogen bonds between DNA base pairs. And it can chemically modify proteins, which disturbs their secondary, tertiary, and quaternary structures, leading to protein fragmentations, protein aggregation, and resultant loss of function as shown in more detail in 2.4 below.

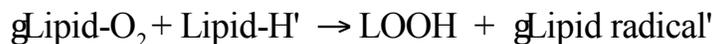
In some instances a series of tandem free radical reactions (a chain reaction) ensues, randomly altering the structure of multiple biomolecules. A good example is the autoxidation of cell membrane lipids. The process is initiated when the lipid (Lipid-H in R 13) comes in contact with hydroxyl radicals and is transformed into a lipid free radical. In the presence of oxygen, the lipid radical is oxidized to a conjugated lipid peroxy radical (Lipid- $\text{O}_2$  in R 14). The lipid peroxy radical can steal a hydrogen atom from a neighboring lipid (Lipid-H' in R 15) to become a lipid hydroperoxide (LOOH in R15), which leaves the neighboring lipid as a free radical (Lipid radical' in R 15). The new radical then continues the process with additional rounds of lipid peroxidations. The radical can also react with proteins causing further damage. Lipid peroxidation products can be detected in blood plasma and have been used as a measure of oxidative stress.



R 13

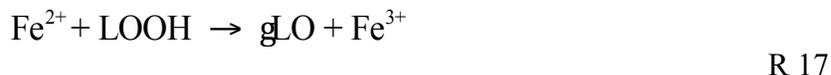


R 14

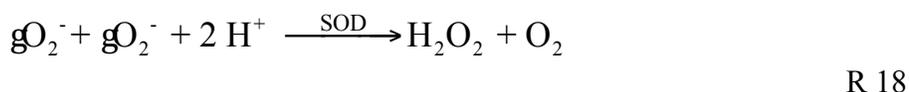


R 15

The presence of redox active metal ions can extend the free radical chain reactions as shown in R 16 and R 17.



Superoxide radicals are also linked to the production of hydroxyl radicals (see ·OH figure 5). This link explains superoxide's toxicity, since, in fact,  $E^0$  of the  $\text{O}_2/\cdot\text{O}_2^-$  couple is only -137 mV (Petlicki and van de Ven, 1998), which means that superoxide by itself is not super reactive as an oxidant. However, superoxide radicals can react with each other to form  $\text{H}_2\text{O}_2$ , catalyzed by the enzyme superoxide dismutase (SOD). The reaction is shown in R 18. The combination of  $\text{H}_2\text{O}_2$  and  $\cdot\text{O}_2^-$  can then yield highly reactive hydroxyl radicals such as presented in R 10 and R 11 above.



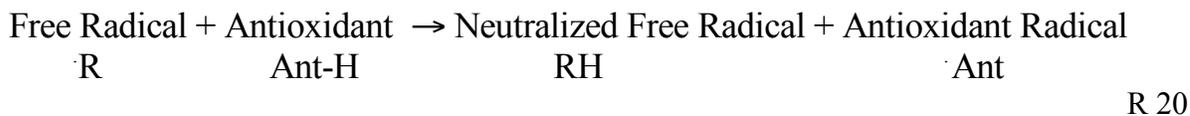
Superoxide can furthermore cause substantial damage since it can react with nitric oxide to form peroxynitrous acid (ONOOH), as shown in R 19. Peroxynitrous acid equilibrates with peroxynitrite ( $\text{ONOO}^-$ ), and both are very powerful oxidants. This reaction might be important near active macrophages where the concentration of ROS exceeds the ability of SOD to dismutate superoxide into oxygen and hydrogen peroxide.



## 2.2 Relevance of Antioxidants for E'

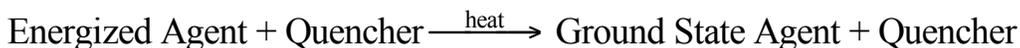
While it may seem that cells are continually under an unrelenting barrage of damaging oxidative attack, it is in fact the case that there are numerous cellular processes that are usually quite effective in protecting against the potential dire consequences of unchecked oxidative attack. Cellular defense strategies against free radical stress include a wide range of chemical agents, which specifically protect against the damaging effects of ROS or other free radicals. These chemical agents are known as *antioxidants*.

Most antioxidants act as *reducing equivalents* that counteract the effects of oxidation as shown in R 20. An abundance of chemically diverse substances fall into this category and examples will be presented in more detail below.



Other antioxidants act as free radical *quenching substances* as shown in R 21. These substances quench, in a physical reaction, energized agents such as singlet oxygen by accepting their energy (Krinsky, 1993). Examples include beta-carotene, a form of vitamin A, and lycopene, a

carotenoid pigment that gives tomatoes their red color.



R 21

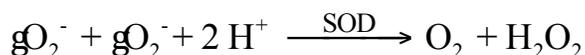
A third mechanism by which antioxidants can act is by *binding metals* such as iron and copper and thus minimizing the metal-dependent production of oxidants as shown in R 22. Examples of antioxidants that act in this way are the iron storage protein ferritin, the di- and trivalent metal ion chelator EDTA, and vitamin E, the most widely known biological antioxidant.



R 22

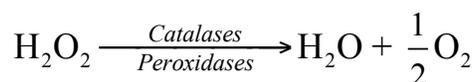
Functionally, antioxidants can be categorized as either *preventive antioxidants*, which capture oxidizing species before they can do damage, or *chain-breaking antioxidants*, which terminate oxidative, free radical chain reactions early in the reaction process. However, many antioxidants fall into both categories. The best known chain-breaking antioxidants are Vitamin E, which is actually a set of 8 related tocopherols, and vitamin C, the essential nutrient ascorbate. These substances can react with peroxy radicals – the most important radical species in the propagation of chain reactions as shown in R 13-17 -, thus terminating reaction chains. Some vitamin E compounds also act as preventive antioxidants by coordinating with heme-iron and upregulating other preventive antioxidants such as ferritin.

Structurally, antioxidants can be categorized as enzymes or non-enzymes. The most important *enzymatic antioxidant* network is the combination of superoxide dismutases (SOD), catalase, and peroxidases. Acting in tandem, SODs first catalytically convert superoxide into oxygen and hydrogen peroxide (R 23). In humans SOD isoforms exist in mitochondria, in the cytoplasm, and extracellularly. The mitochondrial form is a manganese containing glycoprotein, the other two forms contain both copper and zinc in their reactive centers.



R 23

Subsequently, catalases or peroxidases rapidly convert hydrogen peroxide or other peroxides into ground state oxygen and water (R 24). These enzymes can use various transition metals at their active sites. For example,  $Fe^{3+}$  in the form of heme is used to react with  $H_2O_2$  to generate an oxyferryl porphyrin radical cation. Other schemes for enzyme redox catalysis are thiolate-dependent reactions (from  $S^-$  to  $SOH$ ) such as in the case of peroxiredoxins, and selenocysteine-dependent reactions (from  $Se^-$  to  $SeO^-$ ) such as in the case of glutathione peroxidases. Either way, the thiol-selenol interchanges result in the reduction of  $H_2O_2$  to  $H_2O$  or in the reduction of other peroxides ( $ROOH$ ) to their respective hydroxyl compounds ( $ROH$ ) (Stone and Yan, 2006).



R 24

Additional examples of enzymes that are important in antioxidant process are glutathione and thioredoxin reductases. They are presented below in 2.3, together with glutathione and thioredoxin, since their function is to maintain their substrates in the reduced state, in which they

are effective antioxidants.

*Non-enzymatic antioxidants* include, in addition to the already mentioned vitamins, various hormones. One example is melatonin, which is produced in the pineal gland and controls the body's internal clock. Thiols, the compounds with a reactive sulfhydryl (SH) group, are additional highly important non-enzymatic antioxidants. The important cellular redox regulators glutathione, thioredoxin, and glutaredoxin are thiols and are discussed in 2.3 below. Although the list of other non-enzymatic antioxidants could be greatly extended, only three more substances will be named here: Coenzyme Q, a vitamin-like molecule that acts as essential cofactor for various enzymes; bilirubin, a breakdown product of red blood cell heme; and uric acid, a breakdown product of purines (nitrogen containing heterocycles such as adenine and guanine). And of course, there are a wide variety of therapeutic compounds, such as the anti-diabetic metformin, that are employed for their antioxidant capabilities.

Although non-enzymatic antioxidants are widely considered to be health promoting agents, at high concentrations, many of them can damage cells. For instance, uric acid can lead at excessive concentrations to gout and cardiovascular problems, and bilirubin can cause brain and other tissue damage at high concentrations.

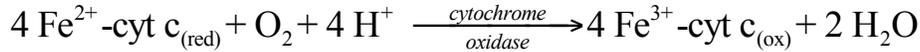
*In vivo*, enzymatic and non-enzymatic antioxidants form complex defense networks. For instance, the negatively charged  $\text{O}_2^-$  molecule that is produced within mitochondria faces a mitochondrial free radical detoxification system which includes various enzymes such as mitochondrial SOD. To minimize the disruption of the mitochondrial membrane by oxidative attacks of its lipids, an additional non-enzymatic regenerative antioxidant system is in place. This system is comprised of the lipophilic compounds vitamin E and coenzyme Q. When vitamin E, becomes oxidized, it is recycled by coenzyme Q, which itself is regenerated by the normal reactions of the respiratory chain (see discussion of R 25 below), in which it acts as a carrier of reducing equivalents (Smith et al, 2003).

Finally it is important to note that in spite of the potentially toxic effects of oxygen discussed above, normal mammalian metabolism, and in fact most cell survival, is impossible without the consumption of substantial quantities of oxygen. In this role, oxygen is the ultimate oxidant in a tandem series of redox reactions that convert reduced dietary energy sources to metabolic energy. Reaction 25 summarizes the overall reaction for oxidation of glucose to carbon dioxide and water. In this reaction the chemical form of energy produced is adenosine triphosphate (ATP) at a ratio of about 34 molecules of ATP formed per molecule of glucose oxidized.



R 25

Other dietary sources such as carbohydrates, lipids, proteins, and nucleic acid can enter the pathways that underlie R 24. These pathways are composed of a series of individual reactions in which electron carriers undergo reversible redox reactions to allow for a stepwise release of energy. The carriers include flavoproteins, cytochromes, iron-sulfur proteins, copper compounds, and coenzyme Q. The main oxygen requiring redox reaction that is essential for the ATP production is the terminal reaction of a mitochondrial process known as *oxidative phosphorylation* shown in R 26. Without oxygen the process is completely inhibited and no ATP can be formed.



R 26

The oxygen requiring step is catalyzed by an enzyme complex known as cytochrome oxidase, or complex IV, which catalyzes the transfer of four electrons. The electrons originated from reduced food molecules and are carried by four iron containing cytochrome c (Fe-cyt c) molecules, to one O<sub>2</sub> molecule.

### 2.3 Relevance of Major Cellular Redox Couples for E'

As mentioned in the last section a cell's antioxidant enzymes are supported by a wide variety of reducing agents that rapidly quench free radicals and thus terminate propagation of free radicals without the intervention of enzymes. We already mentioned several of these non-enzymatic antioxidant reagents. Here, we discuss the endogenous, widely distributed and biologically highly important redox compounds *glutathione*, *thioredoxin*, and *glutaredoxin*.

The standard reduction potentials E<sup>0</sup> of the glutathione, thioredoxin, and glutaredoxin redox couples are between -220 mV and -280 mV, indicating their comparatively robust tendency to donate electrons to other compounds. In these compounds the active redox moieties are cysteine sulfhydryl (SH) residues. When the reduced (SH) forms of these compounds, called thiols, become oxidized, a disulfide (S-S) bond is generated. As shown in figure 6 the SH/ S-S exchange is readily reversible.

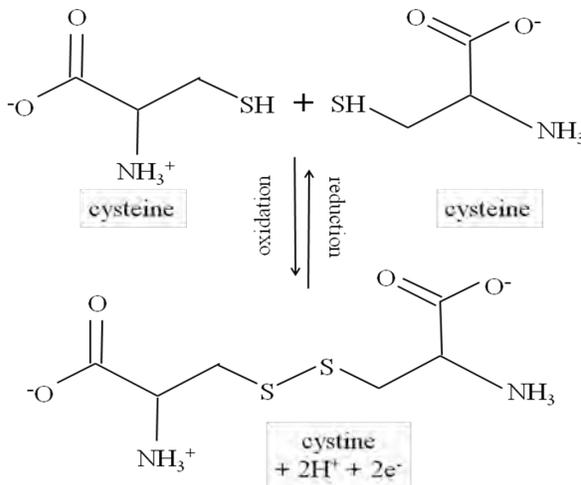


Figure 6: Thiol-disulfide exchange mechanism. Two cysteines become oxidized to cystine, a disulfide, in a reversible manner.

The reversible thiol-disulfide exchange is a central chemical theme in biology. It is used to influence the cellular redox set point via the actions of glutathione, thioredoxin and glutaredoxin. It is part of the active sites of many redox enzymes such as ribonucleotide reductase, which controls the cellular concentration of the DNA building blocks deoxyribonucleotides. It is the mechanisms of action of glutathione and thioredoxin reductases, which catalyze the reduction of disulfide to thiol in glutathione and thioredoxin as shown below in R 28 and R 34. The formation of disulfides bonds is very important for the structural and functional maintenance of most

proteins as discussed in 2.4 below. Furthermore, thiol redox control is an important way to regulate redox sensitive proteins as presented in 3.1 below.

Major redox components using the thiol-disulfide exchange mechanism are arranged in self-sustainable redox networks as shown in figure 7. The left side of the figure shows the glutathione and glutathione-dependent systems, the right side depicts the thioredoxin and thioredoxin-dependent systems as explained in the following.

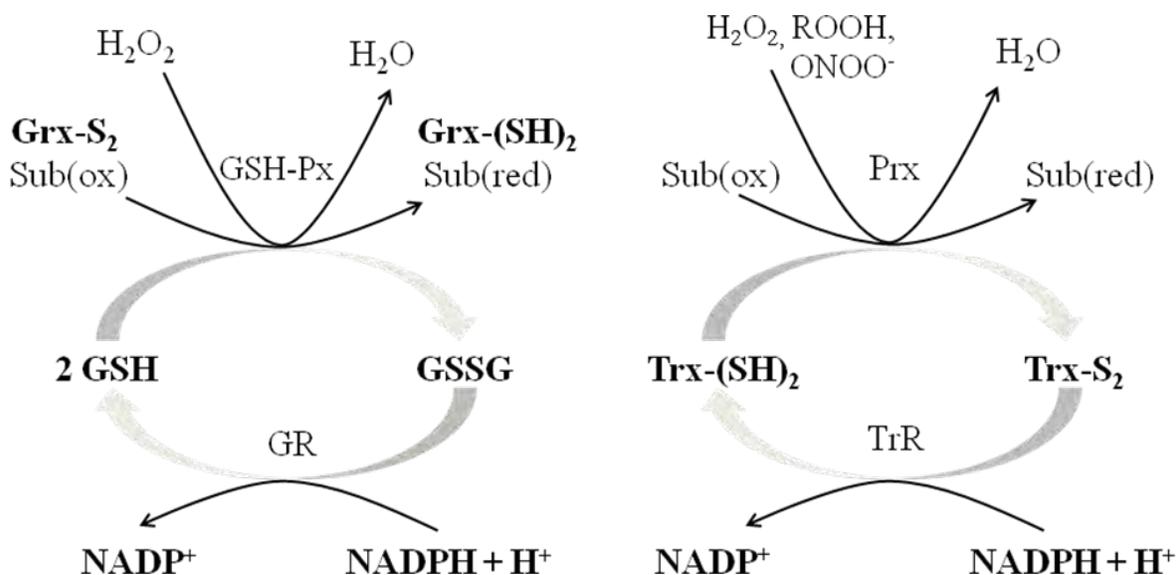
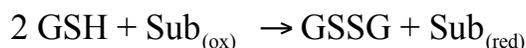


Figure 7: The glutaredoxin and thioredoxin systems in comparison. The glutaredoxin system contains red and ox glutaredoxin (Grx-(SH)<sub>2</sub>, Grx-S<sub>2</sub>), red and ox glutathione (GSH, GSSG), red and ox nicotinamide adenine dinucleotide phosphate (NADPH, NADP<sup>+</sup>) and glutathione reductase (GR). The thioredoxin system contains red and ox thioredoxin (Trx-(SH)<sub>2</sub>, Trx-S<sub>2</sub>), NADPH/NADP<sup>+</sup>, and thioredoxin reductase (TrR). The actions of glutathione peroxidase (GSH-Px) are coupled with the glutathione system, the actions of thioredoxin peroxidase (= peroxi-redoxin, Prx) are coupled with the thioredoxin system. H<sub>2</sub>O<sub>2</sub>: hydrogen peroxide, ROOH: hydroperoxides, ONOO<sup>-</sup>: peroxynitrite.

*Glutathione* (GSH, figure 7) is a tripeptide composed of the amino acids glutamate, cysteine and glycine. Within cells, the cysteine sulhydryl (SH) group of reduced glutathione molecules (GSH) is used to reduce substrates such as oxides and peroxides (Sub<sub>ox</sub> to Sub<sub>red</sub>) and is thus converted to oxidized glutathione (GSSG) containing a disulfide bond (-S-S-). This is shown again in R 27.



R 27

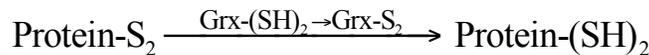
Subsequently, GSH is recycled by *glutathione reductase* (GR, figure 7), a reaction that requires NADPH as shown in R 28.



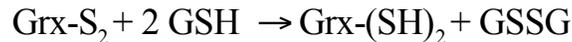
R 28

The ubiquitous presence of NADPH and GR allows cells to maintain a predominantly reducing intracellular environment. Moreover, glutathione is quantitatively the dominant redox buffer, being present in millimolar concentration, as compared to micromolar concentrations of most other redox buffers. Thus, the cellular redox set point can be approximated by measuring the concentrations of reduced and oxidized glutathione and using the Nernst equation (Eq 1) to calculate the prevailing cellular electrode potential.

The glutathione system is connected to glutathione-dependent redox enzymes called *glutaredoxins* (Grx, figure 7). Glutaredoxins are named in analogy to thioredoxins since they both reduce disulfide bonds in cytoplasmic proteins (Fernandes and Holmgren, 2004) as shown in R 29. Grx is recovered by glutathione as shown in R 29.



R 29



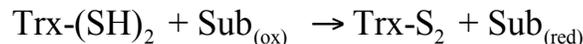
R 30

Figure 7 additionally shows the relationship of the glutathione system to *glutathione peroxidase* (GSH-Px), an antioxidant which we introduced in R 24 above. The enzyme reaction can now be extended by showing that the enzyme catalyzes the destruction of peroxides such as H<sub>2</sub>O<sub>2</sub> by a reaction coupled to glutathione (R 31).



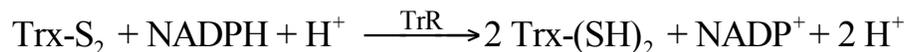
R 31

*Thioredoxin* (Trx-(SH)<sub>2</sub>, figure 7) is a small protein (M.W. 11,700) with two cysteine residues as part of the active site. Like those in glutathione, they participate in SH based redox reactions to form a disulfide bridge. This redox couple is comprised of reduced thioredoxin, Trx-(SH)<sub>2</sub>, and oxidized thioredoxin, Trx-S<sub>2</sub> as shown in R 32.



R 32

Again like glutathione, the oxidized thioredoxin disulfide (-S-S-) is recycled to regenerate Trx-(SH)<sub>2</sub> by reducing NADPH in a reaction catalyzed by *thioredoxin reductase* (TrR, figure 7) as shown in R 33.



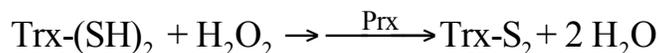
R 33

As with the glutathione couple, the reduced form of thioredoxin is the main form present in the cell. One function of cellular thioredoxin is to reduce the disulfide bonds of other cellular proteins, allowing them to attain their active tertiary and/or quaternary structures. In addition to

controlling protein structure thioredoxin also plays a role as a coenzyme. A well documented example is its coenzymatic activity in conjunction with the enzyme ribonucleotide reductase. In this reaction reduced thioredoxin binds to ribonucleotide reductase and its reducing equivalents are used to reduce the sugar ribose to deoxyribose, which is required for the synthesis of deoxyribonucleotides, the building blocks of deoxyribonucleic acid (DNA).

Recently, a key role has emerged for thioredoxin as a cellular redox signaling molecule. This is often the actual process implied by the term ‘thiol redox control’, which is used to describe the regulation of redox sensitive, cellular activities (see also 3.1 below). As a signaling molecule thioredoxin has been shown to reversibly regulate enzymatic activity as well as the activity of genomic transcription factors and thus gene expression. In addition to being an important intracellular regulator, thioredoxin can also be exported from the cell to act as a cytokine or chemokine (a cellular communication molecule) with cytoprotective roles. The most recent discovery is thioredoxin’s role in activating ion channels and hence regulating communication between extra- and intracellular compartments (Zu et al., 2008).

Just as the glutathione system involves additional glutathione-dependent components such as GSH-Px, the thioredoxin system includes additional thioredoxin-dependent enzymes. Figure 7 shows one such enzyme, *thioredoxin peroxidase* (Prx), also called *peroxiredoxin*. It reacts with peroxides and reduced thioredoxin to form water and oxidized thioredoxin as shown in R 34.



R 34

We have focused our attention thus far on ROS and cysteine related redox species and only considered pyridine nucleotides as a reservoir of reducing equivalents that is used to recycle oxidized cysteines and maintain redox homeostasis. However, it is important to add that the pyridine nucleotides, *NADPH/NADP<sup>+</sup>* and *NADH/NAD<sup>+</sup>*, are highly mobile carriers of reducing equivalents and as such are central players in the energy economy of a cell. In this capacity they shuttle reducing equivalents from high energy nutrient sources to numerous biosynthetic, or anabolic, processes as well as to the mitochondria where the vast majority of cellular ATP is generated from reactions driven by NADH (see R 25 above).

The relevance of these observations lies in the fact that perturbations in the cell redox state not only affect cysteine related cell activities but that they impact the total energy economy of a cell through effects on the redox energy state of the pyridine nucleotides. Examples of these kinds of interactions can be found in cell cultures that are confluent and energy/nutrient-deprived where it seems that GSH is used to reduce *NAD<sup>+</sup>* to *NADH*, which can then be used to generate ATP (Attene-Ramos et al., 2005). This does not happen in growing cell cultures where *NADH* receives its high energy reducing potential from nutrients and carries it to mitochondria, where it is used to drive the production of ATP. Additionally, the pyridine nucleotide link between the cellular redox state and the cellular energy state might be especially important in tissues that are highly sensitive to oxygen levels such as pulmonary arterial smooth muscles (Wolin et al., 2007) and the brain (Hyun et al., 2006).

Historically, bioscientists have almost exclusively viewed the role of pyridine nucleotides to lie in maintaining energy metabolism and redox homeostasis. Recently, however, a new level of complexity emerged when it was found that pyridine nucleotides also act as signaling molecules

by binding, in non-redox reactions, to intracellular and extracellular proteins where they help regulate a wide variety of other non-redox cell functions. These include posttranslational protein modification and other cell signaling pathways. This clear, but complex, coupling between a cell's homeostatic redox state and a multiplicity of other central cell functions lends strong credence to the notion that redox homeostasis in one way or another plays a much bigger role in the behavior and viability of cells than previously thought.

In the preceding we have tacitly assumed that all of the constituents of all of the reactions we discussed were readily available to react with each other so that all redox reactions were in an electrochemical steady state and that redox related enzymes had ready access to all of their substrates. In fact however, some redox couples and redox related enzymes are restricted to specific cell compartments including mitochondria, the endoplasmic reticulum, lysosomes, or the extracellular milieu, with the result that the homeostatic redox set point can differ substantially between any two compartments.

For instance, in the *endoplasmic reticulum*, GSSG is in higher concentration than GSH, resulting in a more oxidizing environment compared to the cytosol (Bánhegyi et al., 2007). The more oxidizing environment of the ER favors the formation of the disulfide bonds of secretory and membrane proteins which are formed in the endoplasmic reticulum during the process of protein synthesis.

Likewise, the *intra-mitochondrial* homeostatic redox state also seems to be shifted towards the oxidized state (Ježek and Hlavatá, 2005). This may be an evolutionary consequence of the fact that the  $E^{0'}$  of mitochondrial, electron transport redox couples is intermediate between the  $E^{0'}$  of nutrient couples and that of the  $O_2/H_2O$  couple which is the final destination of reducing equivalents derived from nutrients. In any event, the relatively oxidizing intra-mitochondrial environment seems to be generated, at least in part, by reactions that regenerate reduced thioredoxin, using GSH-derived reducing equivalents. This GSH, required for regenerating reduced mitochondrial thioredoxin, is readily obtained from the cytosol since GSH can move across the mitochondrial membrane (Chen et al., 2000). However, the GSSG generated within the mitochondrion, is impermeable to the mitochondrial membrane and tends to accumulate, resulting in a high intra-mitochondrial ratio of GSSG to GSH.

The GSH-based cross talk between the cytosolic and mitochondrial glutathione compartments exemplifies the role of *compartmentalization* of redox reactions and the complexity of the processes that maintain a cell's homeostatic redox state. The complexity alluded to here may be just a hint of what is in store since it has recently been discovered that in humans there are 21 thioredoxin reductase splice variants (Rundlöf et al., 2004). The latter foreshadows a complexity of organismal redox homeostasis that has up to now been unanticipated and remains to be fully understood (Smith, 2005).

## 2.4 Role of $E'$ in Protein Structure and Function

It is well known that protein function is dependent on the three dimensional, tertiary or quaternary, state of a protein and that the tertiary and quaternary structure can be - at least subtly, and sometimes dramatically - modulated by chemical and physical influences of the environment

on every atom in the protein. The most well studied *physical* effects are the molecular fragmentation induced by ionizing radiation and the denaturing effects of heat. In the *chemical* sphere, the effect of  $H^+$  concentration on pH sensitive moieties of a protein's constituent amino acids can be considered the best documented of all reactions that modify a protein's three-dimensional structure. However, it is increasingly well recognized that the *redox set point*  $E'$  of a biological system can sensitively, reversibly, and effectively modify protein structure and thus regulate a protein's biological activity. In the biological realm, these redox sensitive structural events are mainly a consequence of the effect of changes in the redox environment which alter the redox state (S-S or SH) of cysteine residues in the protein (see figure 6). Other amino acid residues such as arginine are also influenced by the redox environment but in this discussion we focus on the role of cysteine's redox state in determining protein structure keeping in mind that the discussion applies equally to other redox sensitive moieties in proteins.

The covalently bonded sequence of amino acids in a protein is genetically determined, and this sequence is referred to as the *primary structure* of the protein. Ultimately all properties of a protein are determined by this sequence. As a consequence of hydrogen bonds that form between contiguous amino acids the peptide chain assumes one of a number of geometric forms, the most well known being the  $\alpha$ -helix and  $\beta$  structure. These forms are referred to as a protein's *secondary structure*. Lengths of secondary structures such as the  $\alpha$ -helix are often interrupted by loss of that structure which is induced by amino acids that cannot form hydrogen bonds with their neighbors. These amino acids, exemplified by proline, are known as *structure breakers*. At points where structure breaking amino acids are present the protein becomes especially flexible and can fold into many different forms and shapes. These folded shapes are known as a protein's *tertiary structure*, the most easily visualized tertiary structure being a globular or spherical form. Finally, two or more such proteins with their individual tertiary structure can interact to form a protein complex which is known as the *quaternary structure* of the protein. The most well studied quaternary structure is that of hemoglobin which is normally composed of 4 similar globular proteins.

*Protein folding* is thermodynamically determined by the protein's minimum free energy state and the most structurally stable state of the protein. As an example of how  $E^0$  can determine the structural state of a protein, a protein is considered that contains two cysteine residues located on two different lengths of  $\alpha$ -helix, and in which, when folded, the cysteine residues are juxtaposed. If the  $E'$  of the environment is permissive, the cysteine residues will form covalent disulfide (S-S) bonds which will strongly stabilize that particular folded state of the protein. When the lengths of secondary structure are brought close to each other by the covalent disulfide link additional intra-protein bonding occurs. The bonds that form include salt bridges between acid and basic amino acid side groups, hydrophobic bonds and, perhaps most important, a large number of hydrogen bonds. All of these bonds will further lock the protein into a structurally stable form. On the other hand if the  $E'$  is not permissive to oxidation of SH residues, S-S links will not form and the protein will be free to take up a folded shape that is determined by the formation of weaker bonds such as salt bridges, hydrophobic forces and hydrogen bonds. It is highly unlikely that both the disulfide form and the sulfhydryl form of the protein will have similar structure or equivalent biological activity, so it is clear that redox potential can regulate a protein's biological activity. If the protein first exists in the oxidized (S-S) form and the environmental redox potential changes so as to reduce the disulfide bond and to generate free SH residues, hydrogen and other bonds will tend to maintain a folded state. This minimum energy state is a very close,

but not identical form to the disulfide bonded form of the molecule. Again it is not likely that the protein will retain the full biological activity of the disulfide bonded form but it may be at least partially active.

In the scenarios presented above we have illustrated the notion that the redox state of a cell can dramatically modulate the structure and activity of a redox sensitive protein. Or, that it can sensitively modulate protein activity with the resultant activity depending on the new, minimum energy, structural state, of the molecule. The latter of course is determined by the remaining bonding forces including hydrogen bonds. Since ROS can influence the redox set point of a biological system (see 2.1 above), it seems apparent now that changes in the level of ROS can modulate protein activity through its effect on a systems redox set point.

### **III Therapeutic Relevance of the Redox Set Point E'**

#### **3.1 ROS Signaling as Basis for Redox Therapy**

We have introduced the cellular redox set point, E' and seen that homeostasis of E' is a key feature of life, obtained by antioxidants that balance cellular oxidative metabolism and production of ROS. In this current section, we stress the second role of ROS, in which they are used to benefit the cell, under normal conditions and in therapy.

The essential role of ROS in protecting the body against microbial infections by contributing to the microbicidal activity of phagocytic white cells (see R 9 above) has been known for some time. Over the years, it has become increasingly evident that ROS additionally act as mediators of host defense by participating in cell signaling of various immune cells, by activating transcription factors of immune cells, and by altering cell signals that affect growth, differentiation, and cell death of immune cells. Knowledge about similar roles of ROS in many other cell types arises at an increasing rate and leads to speculations of ROS as ubiquitous cell modulators (Filomeni et al., 2005). The mechanisms by which oxygen and reducing equivalents transmit information within cells are called *ROS signaling*.

To understand the special nature of ROS signaling, the established process of receptor signaling is briefly introduced. The first step in this process is that cells sense signal molecules, called ligands (L, figure 8), with distinct proteins, called receptors (R, figure 8). Typically, ligands and receptors have the ability to modify their three-dimensional structures so that they can form, what has been alluded to as an induced lock and key structural relationship that facilitates tight bonding between them. This is called the *ligand receptor complex*. The complex is stabilized by non-covalent ionic, hydrogen, or van der Waals bonds in a reversible process. How long and how well the complex stays together – measured by the binding affinity – determines the duration and extent of the biological response. The biological response is mediated by a cascade of biological reactions, known as the *signal transduction cascade*. These cascades, symbolized in figure 8 by simple arrows, can begin on the exterior surface of the cell membrane when extracellular ligands bind to membrane-localized receptor proteins; or it can happen inside the cell, in the cytoplasm or the nucleus, when intracellular messenger ligands interact with signal transduction proteins leading to the activation of transcription factors.

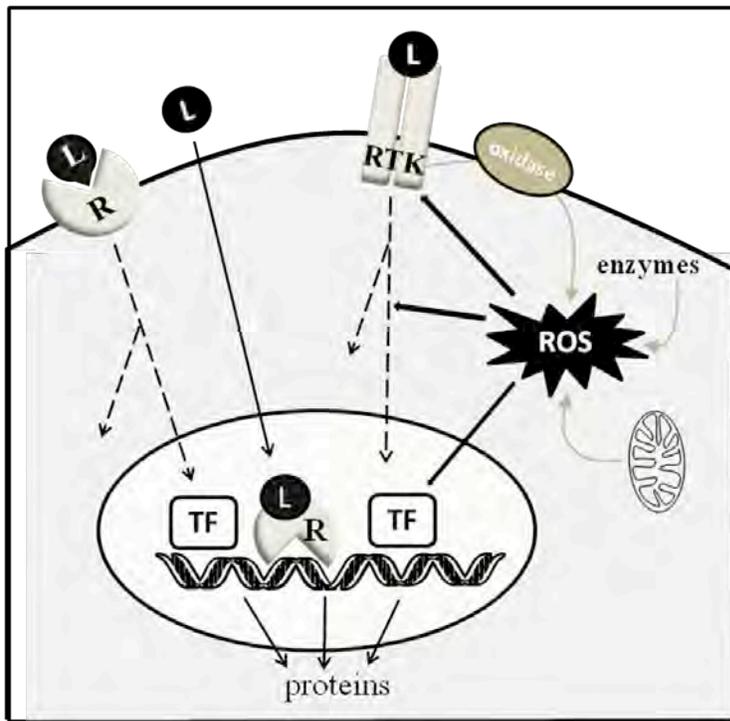
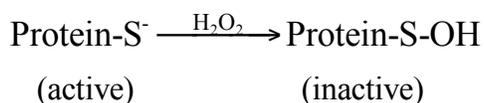


Figure 8: Cell signaling mechanisms. Traditional signal molecules and reactive oxygen species (ROS) signal molecules are shown in comparison. For details, see text. L: ligand, R: receptor, RTK: receptor tyrosine kinase, TF: transcription factor.

Examples of typical ligands that can form a ligand receptor complex are growth factors, hormones, neurotransmitters, and second messenger molecules. It has now been discovered that ROS modulate many membrane-associated and intracellular proteins and could therefore be considered as ligands. This is best described in prokaryotes (D'Autréaux and Toledano, 2007). For example, in *E. coli* bacteria  $H_2O_2$  activates a nuclear transcription factor, called Oxy receptor (OxyR), which ultimately leads to increased resistance against oxidative stress in the bacteria.  $H_2O_2$  activates OxyR by oxidizing specific cysteine residues that are present in the reactive center of the protein, forming disulfide bonds. The process is reversed by glutaredoxin 1 to reconstitute the resting state of the receptor. Other examples have been described, and in all cases, the ROS ligand activates the receptor by reacting with specific molecules or atoms of the receptor, which is different from the temporary formation of the protein-receptor complex described above.

The specificity of the interaction between ROS and the receptor occurs because specific ROS react preferably with specific biological targets. For instance,  $H_2O_2$  is known to react primarily with certain active site cysteine residues such as present in OxyR. These special active site cysteines have an abnormally low acid dissociation constant,  $pK_a$ , when compared to most cysteines, which have an alkaline  $pK_a$  (Mössner et al., 2000). This means, that at physiological pH, active site cysteines are mainly in the dissociated state and their deprotonated sulfur can undergo ROS reactions, which alters the activity state of the cysteine containing protein, in R 35 from an active to an inactive state.



R 35

Active site cysteines are also part of mammalian biomolecules. They are for example part of the thiol groups of glutathione peroxidase and thioredoxin peroxidase (see R 30 and R 33 above), which means that these thiol peroxidases are not only redox transducers, as described above (see R 23), but that they also participate in ROS signaling.

Mammalian ROS signaling proteins are mostly molecules that were previously recognized in another context but that are now understood as being able to specifically react with certain ROS. They include compounds that participate in homeostatic redox reactions, signal transduction, and gene activation as indicated in figure 8. Well known examples of ROS-modulated transcription factors are activator protein 1, AP-1, and nuclear factor-kappa B, NF-κB, (Zhou et al., 2001; Forman et al., 2002). The best studied signal transduction proteins that are intracellular targets of H<sub>2</sub>O<sub>2</sub> are various thiolate-dependent phosphatases (TDPs) such as protein tyrosine phosphatases (PTPs). H<sub>2</sub>O<sub>2</sub> reacts with PTPs at a rate constant comparable to other enzymes and these oxidative reactions lead to the transient inhibition of the PTPs (Rhee et al., 2000; Rhee, 2006). Inhibition of PTPs means for the cell that tyrosine residues of other signaling proteins that have been phosphorylated by protein kinases remain longer in their phosphorylated state. The process is reversible in that the oxidized PTPs gradually return back to their reduced, or active, state, which terminates the signal transduction.

PTP signal transduction is a part of signaling pathways that are activated by ligand binding to catalytic tyrosine kinase receptors (RTK, figure 7). The ligands of RTKs include growth factors (e.g. EGF, PDGF, IFG-1) and hormones (e.g. insulin). The binding of these ligands not only causes the activation of the receptor and thus the initiation of consequent downstream events, but also leads to a dramatic upregulation of Nox and other oxidases. These oxidases produce ·O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub>, and other oxidants that act as signal transduction modulators, for instance by binding to PTPs (Ree et al., 2000). It turns out that many growth-factor induced signaling pathways require ROS for optimal signaling (Stone and Yang, 2006).

The most attractive and best studied ROS signaling molecule is clearly H<sub>2</sub>O<sub>2</sub>. Interestingly, the H<sub>2</sub>O<sub>2</sub> produced by RTK-associated oxidases is released into the extracellular space or into formed vacuoles, from where it can diffuse into the cytoplasm. It is estimated that about one tenth of the extracellular H<sub>2</sub>O<sub>2</sub> reaches the cytoplasm and that intracellular concentrations between 20 nM and 1 μM can act as signaling ligands (Rhee et al., 2000). H<sub>2</sub>O<sub>2</sub> primarily (not exclusively) reacts with cysteine molecule, in the form of active site cysteines, such as part of thiol peroxidases, and in the form of zinc cysteine centers, such as part of heat shock protein 33. A second signaling candidate is ·O<sub>2</sub><sup>-</sup>, since it has a tendency to react with [Fe-S] clusters. For example, in *E. coli* ·O<sub>2</sub><sup>-</sup> specifically reacts with the transcription factor SoxR, which contains a [2Fe-2S] cluster, leading to the transcription of oxidative stress genes (Koo et al., 2003). Recently, it has even been suggested that the highly reactive ·OH acts as signaling modulator after RTK-induced H<sub>2</sub>O<sub>2</sub> is transformed to ·OH by a Fenton reaction as shown above in R 9 (Wong et al., 2008). An increasing number of proteins with tyrosine, tryptophan, cysteine, and glycine residues are being considered as reversible ROS signaling targets.

Many of the long-term biological effects of ROS signaling can be understood as being involved

in the protection of the organisms against oxidative stress (e.g. Ahn and Thiele, 2003) and in the elimination of redox-stressed cells by apoptosis (Simon et al., 2000). However, more and more biological actions that are initiated by redox imbalances cannot be seen primarily as part of the defense against oxidative stress. Examples of these non-stress protective roles are the regulation of exocytosis of insulin granules (Ivarsson et al., 2005) and vasorelaxation caused by redox-activation of the smooth muscle relaxant protein kinase G 1 $\alpha$  (Burgoyne et al., 2007).

When placing ROS signaling in the context of redox homeostasis, it seems evident that the ROS induced protein modifications cannot be seen as separate reactions, but instead as reactions that go hand in hand with the mechanisms introduced earlier that regulate the cell's redox set point  $E^0$ . During evolution of aerobic organisms, the development of redox sensing proteins developed in response to the unavoidable production of ROS and the systems to eliminate them. Since excessive ROS production causes denaturation of proteins and other biomolecules, either directly or indirectly through the life-long accumulation of non-functional ROS-damaged macromolecules (Schriner, 2005), redox-sensitive signaling pathways are closely tied to cellular oxidative stress responses. However, there is an emerging understanding that small, non toxic, changes in  $E^0$  can cause temporary conformational and functional changes in proteins. These small changes in  $E^0$  can be generated by ROS produced by RTK-dependent oxidases or by up-or down regulation of antioxidants. Antioxidants are not passive bystanders of ROS signaling, but contribute actively through their selective expression, activation, and compartmentalization to create subtoxic ROS concentrations that then act as signal messengers. In this way, ROS seem to be essential co-regulatory signals that fine tune thousands of cellular actions.

### 3.2 Therapeutic Potential of Non-ROS Small Signaling Molecules

Although it has become more and more accepted that small changes in redox homeostasis are normal and important cell signals, there are still many issues to solve. One such issue is that elimination of  $H_2O_2$  by peroxiredoxin (see R 23) is several orders of magnitudes faster than the  $H_2O_2$  reaction with TDPs. This leads to the speculation that, *in vivo*, growth-stimulating nuclear transcription and translation factors are the real targets of  $H_2O_2$  since it has been shown that they respond to  $H_2O_2$  concentrations which are much lower than that necessary for the activation of TDPs (Rhee, 2006). It might be that *reactive nitrogen species* (RNS), not ROS, are the real signaling molecules. RNS are known to react with the same targets as  $\cdot O_2^-$  and  $H_2O_2$ , only much faster. These include Fe-S and Zn-S centers of proteins and cysteine and tyrosine residues (Schöneich and Sharov, 2006). On the other hand, RNS, like ROS, are also known to react with many other biomolecules leading to protein nitrations, and in combination with ROS, to protein oxidations. While it is not the aim of this paper to review the biological role of nitrogen oxides, the presentation of its most famous member, the gaseous molecule nitric oxide, is used to illustrate the therapeutic potential of energized oxygen metabolites.

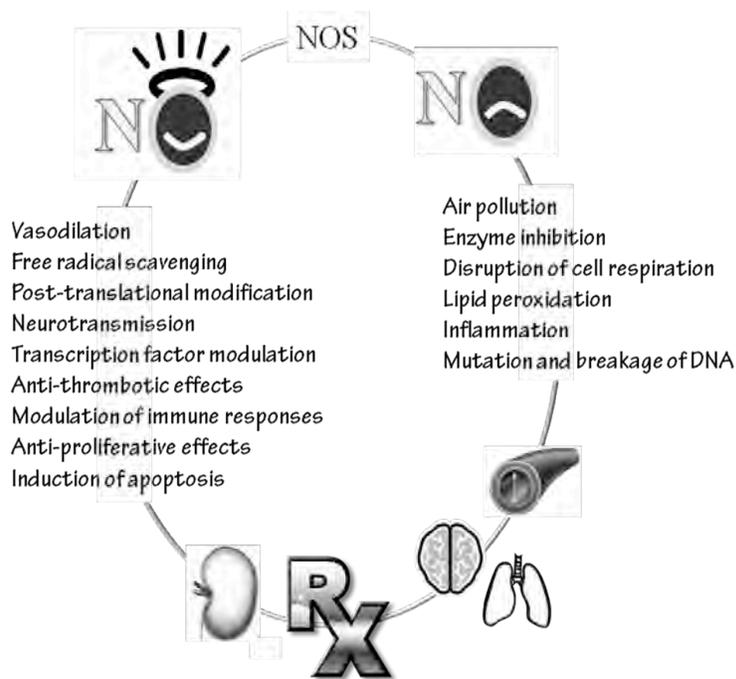
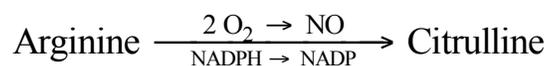


Figure 9: The three images of nitric oxide (NO), as cell toxin (right), as cell modulator (left), and as therapeutic agent (bottom).

As early as the 19th century, nitroglycerin (or chemically more correct glyceryl trinitrate) was used as a therapeutic agent against hypertension, but it took more than another century to discover that nitric oxide ( $\cdot\text{NO}$ ) was generated from the nitroglycerin and that this  $\cdot\text{NO}$  is the chemical responsible for the biological effects of the drug (Napoli and Ignarro, 2003). Nitric oxide is undoubtedly the most famous story of a molecule that was initially viewed to be either biologically irrelevant or perhaps even toxic (figure 9, right side), but that was subsequently discovered to be an important signaling messenger involved in the normal functioning of every human organ. The left side of figure 9 lists some of  $\cdot\text{NO}$ 's cellular functions. The first recognized role of  $\cdot\text{NO}$  signaling was as a vasodilator in a process regulated by  $\cdot\text{NO}$ 's role as a vascular smooth muscle relaxant. Today, many other signaling roles of  $\cdot\text{NO}$  are known and this knowledge is used for therapeutic interventions such as in blood pressure medications, drugs that enhance penile erections, and inhalation therapy for newborns with breathing problems. The medical benefit that derives from the new knowledge is only in its infancy as  $\cdot\text{NO}$  is increasingly being used to help treat disease states such as septic shock, arthritis, kidney disease, stroke, Alzheimer disease and Parkinson disease (figure 9, bottom).

Nitric oxide is produced by NO synthases (NOS) which exist in three isoforms, a constitutive form, an inducible form, and a neuronal form. The specificity of the NO system is mainly determined by the specific gene expression of NOS and NOS inhibitors and by their specific activity and cellular distribution. NOS catalyze the oxidation of L-arginine to citrulline and  $\cdot\text{NO}$ , in the presence of oxygen and NADPH (Lambeth, 2004). The amino acid conversion occurs in two successive reactions that are shown in R 36 in a condensed form.



R 36

There are many similarities between  $\cdot\text{NO}$  and  $\text{H}_2\text{O}_2$ , which makes it tempting to predict a bright pharmacological future for  $\text{H}_2\text{O}_2$ . Like  $\text{H}_2\text{O}_2$ ,  $\text{NO}$  is a relatively long-lived molecule that can diffuse across cell membranes. Like  $\text{H}_2\text{O}_2$ , it prefers to interact with S atoms and metal ions. The reaction of  $\cdot\text{NO}$  with cysteine residues in proteins, called nitrosylation, is an important mechanism of post-translational protein modification that determines protein structure and function. Furthermore,  $\cdot\text{NO}$  modulates iron and zinc homeostasis by interacting with Fe-S and Zn-S clusters in proteins.

$\cdot\text{NO}$  is even more reactive than  $\text{H}_2\text{O}_2$ , and can quickly interact with many other molecules. In fact, it reacts so quickly that it often escapes  $\text{NO}$  scavengers such as oxyhemoglobin and calcium (Margulis and Sitaramayya, 2000). This can cause cellular injury, including the disruption of mitochondrial respiration, the inhibition of enzymes, and the peroxidation of lipids (Beckman and Koppenol, 1996), especially when  $\cdot\text{NO}$  reacts with  $^3\text{O}_2$  and  $\cdot\text{O}_2^-$  to form the reactive compounds  $\text{N}_2\text{O}_3$  and  $\text{ONOO}^-$ , respectively. Despite  $\cdot\text{NO}$ 's potential toxicity, it is nowadays more known as a beneficial cell agent. First,  $\cdot\text{NO}$  can act as an antioxidant that stops free radical chain reactions (Wink et al., 2001). Secondly,  $\cdot\text{NO}$  acts as a beneficial antioxidant by modulating cellular processes that limit oxidative injury.

In this second capacity,  $\cdot\text{NO}$  activates intracellular pathways by reversibly binding to an allosteric site (a site other than the catalytic site) of a target protein, which causes a conformational change of the protein and the activation of a signaling pathway. A common  $\cdot\text{NO}$  target protein is guanylyl cyclase.  $\cdot\text{NO}$  binding to guanylyl cyclase leads to increased production of cGMP, which is a well known regulator of cellular proteins such as protein kinases, ion channels, and phosphodiesterases. When it was discovered that  $\cdot\text{NO}$  also acts in this way in neuronal networks, the traditional view of unidirectional, pre to post-synaptic neuronal transmission had to be extended to include dissolved gases that diffuse freely from cell to cell. Currently,  $\cdot\text{NO}$  is recognized as an important neurotransmitter involved in learning and memory (Hidalgo et al., 2007), and in directing gastrointestinal tract motility. Moreover,  $\cdot\text{NO}$  is an important neuromodulator that can influence the release of almost all other neurotransmitters.

Carbon monoxide (CO) seems to follow  $\cdot\text{NO}$  on “a path of transforming from a toxin to a potential therapeutic.” CO has many similarities to  $\cdot\text{NO}$ . It is a water soluble gas under biological conditions with a similar molecular weight, and it is present in almost all tissues. On the other hand, CO is not a radical and hence is much more stable than  $\cdot\text{NO}$ . The biological functions of CO are also similar to  $\cdot\text{NO}$ . CO is a vasorelaxant, a neurotransmitter, and a regulator of apoptosis. As such, it is generally less potent than  $\cdot\text{NO}$ , thus it was hypothesized that CO may be a cofactor for  $\cdot\text{NO}$  pathways (Hartsfield, 2002). This view is supported by the fact that hemoxygenases (HO), the enzymes that endogenously produce CO, are comparable to NOS in that they have a basal activity which can be upregulated by the same stimuli. In cell culture and animal experiments, an abundance of other therapeutically useful CO effects on many different organ systems have been documented (Hoetzel and Schmidt, 2006).

Human CO data are still sparse. Dose-dependent clinical trials are dangerous since CO binds more strongly to hemoglobin and myoglobin than does  $\text{O}_2$  and hence can cause cardiac arrest, asphyxia, and death. While more data need to be accumulated to better understand the therapeutic potential of CO, it is already without doubt that CO is another small molecule that plays an important role in the newly emerging, seemingly paradoxical, world of oxidative stressors that are also beneficial cell regulators (Ryter et al., 2006). The involvement of the CO system in cell and redox regulation goes even beyond the direct and indirect actions of CO.

When CO is produced by HO through the enzymatic degradation of heme, the two redox active components biliverdin and iron are produced as well. Biliverdin is enzymatically reduced to bilirubin, a very potent ROS scavenger. Iron binds to ferritin, which has anti-oxidant, anti-inflammatory, and anti-apoptotic functions (Dulak and Jozkowicz, 2003).

A third small molecule that has recently generated a great deal of interest is hydrogen sulfide or H<sub>2</sub>S. Like ·NO and CO, H<sub>2</sub>S is a water soluble, colorless, highly toxic gas whose reactions are well known in non-biology chemistry. H<sub>2</sub>S is especially interesting since, unlike ·NO and CO, it does not involve oxygen. H<sub>2</sub>S gives sulfur with its abundant cellular presence -in the form of protein bound Fe-S clusters- a new biological relevance. It remains for future work to unravel which of the many biochemical sulfur reactions are physiologically important (Fontecave, 2006). H<sub>2</sub>S can act, like ·NO and CO, as a vasodilator (Elrod et al, 2007), so that H<sub>2</sub>S might be the way to therapeutically obtain the beneficial effects of ·NO and CO without the risk of causing further oxidative stress. The effect of H<sub>2</sub>S in the vasculature might explain the cardioprotective action of garlic, which is a component of many heart healthy diets.

### 3.3 ROS Therapeutic Strategies

Currently, most clinical attention is focused on the strategy that employs antioxidants to preemptively eliminate ROS before they cause biological damage. Based on excellent *in vitro* evidence showing that antioxidants can eliminate ROS and associated oxidative cell stress, many large-scale clinical trials have been conducted to test the *in vivo* benefit of antioxidant therapy. Up to now, the outcomes of many of these trials have not met the expectations. For instance, a trial in which the impact of high dose vitamin E (i.e. 400 IU/ day) supplements was tested on 9541 participants at risk for cardiovascular events, no positive therapeutic effect could be shown after a mean of 4.5 years (Yusuf et al., 2000). Even worse, a more recent study with an average duration of 7.0 years showed a negative therapeutic effect in that patients receiving vitamin E treatment had a slightly elevated risk of congestive heart failure compared to control patients (Lonn et al., 2005). Likewise, the outcomes of large-scale long-term clinical trials using antioxidants to reduce the risk of brain disorders or trials that used antioxidants in the fight against cancer were similarly ambiguous (Shen et al., 2007). The National Cancer Institute of the US National Institutes of Health summarizes the outcome of five large-scale anti-cancer clinical trials as being inconclusive, with one beneficial, two neutral (no effect and no harm), and two negative therapeutic outcomes (National Cancer Institute, 2008).

To explain the unexpected adverse effects of human trials, it has been proposed that high doses of orally taken antioxidants such as vitamin E lead to an oxidative milieu in which the tested therapeutic acts as oxidant rather than as antioxidant (Linnane et al., 2007). An argument that has been brought forward to explain the lack of beneficial outcomes from the trials is that antioxidants are only expected to work when the redox state E' is disturbed, but that this is usually not the case in people, even if they are at higher risk of getting a disease (Shen et al., 2007). Another argument to explain the lack of positive results especially in trials with elderly people is that it is unlikely to see clinical benefits in patients with chronic diseases by eliminating ROS since there may be many redundant mechanisms that contributed to the development and progression of complex diseases (Fruehauf and Meysken, 2007; Lambeth, 2007). Adequate treatment of complex diseases needs to include strategies to eliminate the underlying causes, the contributing factors, and the complications of disease, and hence, many newer trials are designed

to better address this. Additionally, newer trials typically use multi-antioxidant supplementation to address the fact that antioxidants act as part of complex redox networks and they use lower doses of antioxidants to address the assumption that low doses of ROS are necessary to support normal cellular signaling. Recent outcomes of such modern approaches are promising (e.g. Arnauld et al., 2007).

One lesson to learn from the recent history of ROS-based therapies is that the goal of future therapy cannot be to simply scavenge ROS but instead to modulate existing cellular signal transduction pathways so as to direct cells into a normal or a therapeutically preferred metabolic state. The advantage of such signal transduction modulation (STM) therapy is its specificity. To use the example of cancer, modern STM cancer therapy aims at targeting specific cellular signal transduction pathways involved in carcinogenic tumor growth, in sharp contrast to the dominant cancer therapies, radiotherapy and chemotherapy, which target all dividing cells, including many normal cells. Hence, STM cancer therapy is expected to cause fewer side effects, such as drug resistance, when compared to traditional therapies. Additionally, STM cancer therapy is expected to spare healthy cells such as immune cells which can then initiate the body's natural immune responses against cancer and thus augment the STM therapy.

An example of the way in which redox based strategies might impact STM cancer therapies is found in studies on lung and breast cancers. In these cancers, epidermal growth factor receptors (EGFR) are overexpressed, which leads to excessive activation of the oncogenic Ras signaling pathway(s) and to oncogenic cell proliferation. As described above in 3.1, ROS such as  $H_2O_2$  are necessary for the normal progression of EGFR linked signaling pathways (Soo Bae et al., 1997). This suggests that reduction of  $H_2O_2$ , for example by inhibiting oxidases, has the potential to result in down regulating EGFR signaling and reducing tumor growth. This might directly provide a ROS related cancer therapy or it could be that down regulation of EGFR activity by depleting available  $H_2O_2$  would have a positive synergy with the anticancer activity of chemotherapeutic drugs like erlotinib and gefitinib which inhibit the tyrosine kinase enzyme activity of the epidermal growth factor receptor (Cascone et al., 2006).

Cancer therapy is only one example of an abundance of therapies that could benefit from ROS signaling regulation. If it turns out that ROS are indeed ubiquitous signal transduction modulators involved in the normal regulation of every human cell, the therapeutic possibilities appear to be endless. All enzymes and proteins involved in the pathways of redox signaling are potential drug targets (Seifried et al., 2007). And this therapeutic potential is widely recognized, in the areas of cardiovascular diseases and diabetes (Jay et al., 2006), brain diseases (Faraci, 2006), musculoskeletal disorders (Ferreira and Reid, 2008), and aging (Yildiz, 2007), to name just a few.

Despite this awareness, it is unlikely that within the next decade ROS therapy will play a significant role in human therapy. The limitation for the fast implementation of small molecule signaling therapy is mainly our limited knowledge on how individual reactive species and gases function as part of complex biological networks. Every molecule mentioned in this article has been discussed at one or another time as potentially being troublesome and beneficial to human health. But it is inevitable that ROS signaling has to be taken into account for the design of any new disease intervention and for finding the explanation of existing, mechanistically yet unexplainable, therapies. The near future will reveal if research in this field is at the brink of a new therapy era, in which redox and energy transfer therapies modulate and expand the realm of existing therapies.

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