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Antioxidant Therapeutics: Pandora's Box

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Abstract

Evolution has favored the utilization of dioxygen (O_2) in the development of complex multi-cellular organisms. O_2 is actually a toxic mutagenic gas that is highly oxidizing and combustible. It is thought that plants are largely to blame for polluting the earth's atmosphere with O_2 due to the development of photosynthesis by blue green algae over 2 billion years ago. The rise of the plants and atmospheric O_2 levels placed evolutionary stress on organisms to adapt or become extinct. This implies that all the surviving creatures on our planet are mutants that have adapted to the "abnormal biology of O_2." Much of the adaptation to the presence of O_2 in biological systems comes from well coordinated antioxidant and repair systems that focus on converting O_2 to its most reduced form, water (H_2O) and the repair and replacement of damaged cellular macromolecules. Biological systems have also harnessed O_2's reactive properties for energy production, xenobiotic metabolism, host defense, and as a signaling messenger and redox modulator of a number of cell signaling pathways. Many of these systems involve electron transport systems and offer many different mechanisms by which antioxidant therapeutics can alternatively produce an antioxidant effect without directly scavenging oxygen-derived reactive species. It is likely that each agent will have a different set of mechanisms that may change depending of the model of oxidative stress, organ system, or disease state. An important point is that all biological processes of aerobes have co-evolved with O_2 and this creates a Pandora's Box for trying to understand the mechanism of action(s) of antioxidants being developed as therapeutic agents.

The Abnormal Biology of Oxygen

O_2 is unique in that it is a relatively stable free radical with two unpaired electrons that have parallel spins. This feature restricts O_2 to accept electrons one at a time and is used as an electron acceptor in electron transport chains that are abundant in biological systems. The partial reduction of O_2 leads to a cascade of oxygen-derived species that contribute to damaging cellular macromolecules, tissue injury, dysfunction, and disease (Figure 1). All higher eukaryotes require oxygen as the terminal electron acceptor for mitochondrial ATP generation. The mitochondrial electron transport chain accepts electrons from either NADH or $FADH_2$ and passes them on to the terminal cytochrome oxidase which collects electrons on each of its four iron-heme subunits which are added sequentially to O_2 to form H_2O [1]. The iron-heme subunit is actually a naturally occurring metalloporphyrin and an important cofactor in many different electron transport systems. This is a very efficient process, but a small percentage of the electrons leak as partially reduced O_2 species as superoxide (O_2^-)

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and hydrogen peroxide (H_2O_2) [2]. Although both O_2^- and H_2O_2 are reactive species, it is thought that most of the oxidative damage to cellular macromolecules occurs through additional reactions of these molecules with transitional metals giving rise to the formation of hydroxyl radicals, a three electron reduction of O_2 [3]. Another electron transport system that uses NADPH and produces a prominent oxygen derived free radical, nitric oxide (NO), is nitric oxide synthase (NOS) [4]. Like many of these types of oxidoreductases, NOS can also produce O_2^- under certain conditions [5]. O_2^- and NO rapidly react to form peroxynitrite ($ONOO^-$) [6]. $ONOO^-$ is a strong oxidizing and nitrating species capable of damaging cellular macromolecules [7]. The haloperoxidases are oxidant generating systems that utilize H_2O_2 to generate another series of oxygen-derived reactive species known as halous acids (HOX) which are produced for host defense [8–10]. It is ironic that what often separates a pro-oxidant from an anti-oxidant is the efficiency at which the agent or process converts O_2 to water. Indeed, biologic systems use very similar process to either generate or scavenge partially reduced oxygen species. There are a number of electron transport systems that generate or leak O_2^- and H_2O_2 (Table 1). An example is the use of electrons from NADPH by NADPH oxidases (NOXs) to generate O_2^- which rapidly dismutates spontaneously or enzymatically to H_2O_2 [11]. This brings up two important points to consider: 1) that wherever there is O_2^- there will be H_2O_2; and 2) superoxide dismutases (SODs) still leave behind H_2O_2 and thus can't function alone as an antioxidant and only act as antioxidants in coupled processes that leads to the formation of H_2O.

Antioxidant systems that regulate cellular H_2O_2 levels also rely on NADPH [12] (Table 1). Both the glutathione peroxidase (GPx) and peroxiredoxin (Prx) systems rely on glutathione (GSH) and thioredoxin (Trx), respectively, to scavenge H_2O_2 and their oxidized products are recycled by glutathione and thioredoxin reductases that utilize NADPH, respectively (Figure 2). Biological systems try to avoid the addition of an electron to H_2O_2 which can happen with reduced transitional metals or iron-heme complexes. Transitional metals are kept in less reactive bound states by metal-binding proteins and free iron-heme groups are tightly regulated by heme oxygenase, which ironically is another NADPH dependent electron transport system [13]. Although iron-heme groups are often thought to be involved in pro oxidant reactions they are utilized by catalase to perform a two electron dismutation of H_2O_2 to produce O_2 and H_2O [14]. Many of the one and two electron transport systems can operate as either oxidant generating or scavenging systems. Any process that augments or inhibits these systems can change the steady-state level of oxygen derived reactive species. As one can see, in biological systems there is not such a clear divide between processes that lead to either a pro-oxidant or antioxidant outcome. This is also true for the development of antioxidants as therapeutic agents that are thought to mimic the biological systems.

Role of Oxygen-derived Reactive Species in Oxidative Stress and Cellular Signaling

The term "oxidative stress" is probably one of the most vague and often abused terms in science second only to the term "inflammation." Often both terms are used together in a chicken and the egg type of scenario. Typically oxidative stress implies that there is an imbalance between oxidant production and antioxidant and repair defenses resulting in the increased steady-state levels of oxidized cellular macromolecules [15]. Many antioxidant agents are often validated by blocking the increased steady-state levels of oxidized cellular macromolecules in animal models involving oxidative stress [16–33]. This popular view does not account for why adaptive responses have failed which is probably more important mechanistically than the simple fact that steady-state levels of oxidized cellular macromolecules are increasing. An important issue is whether the oxidation of biological molecules play a causative role in the pathogenesis of disease or is simply a consequence of

the disease process [34, 35]. Although this point is continually raised when antioxidants fail in clinical trials, it is important to consider that successful treatment will require knowing the exact mechanism behind the failure to adapt in order to pick the appropriate antioxidant to intervene. It is this critical gap in our knowledge that has handicapped the successful development of antioxidants as therapeutically useful agents. Aging is an excellent example of a natural process that results in a loss of adaptive responses leading to elevated steady-state levels of oxidized cellular macromolecules [36]. Aging is a common lynchpin of many human diseases and yet we still don't have a clear picture why adaptive responses slowly fail during the aging process [35]. Many examples of adaptive responses to short oxidant exposures have been described such as brief hyperoxic exposures that substantially raise the burden of oxidants in the lungs but also produce a rapid increase in the antioxidant defenses far above the oxidant burden and are protected against further oxidant exposures [37]. Since most oxidant exposures evoke adaptive responses, many antioxidant compounds may actually have paradoxical effects by blocking adaptive responses which invoke a more coordinated antioxidant protection than provided by the antioxidant agent. The so called "bell shaped dose response curves" observed with many antioxidant agents are likely a consequence of this phenomena. An example of an antioxidant producing paradoxical effects in humans have been documented and occurred with chronic β-carotene supplementation in cigarette smokers which had the paradoxical effect of increasing cancer risk [38–41].

Many of the oxidant-induced adaptive responses are triggered by oxygen derived reactive species directly or indirectly impacting cell signaling pathways. These events have been best studied in bacteria where specific transcriptional factors oxyR and soxRS have been identified [42, 43]. OxyR is activated by H_2O_2 and induces a number of antioxidant and DNA repair genes products [44, 45]. It is interesting to note that in the soxRS system, the soxR protein that senses the oxidant signal contains an iron-sulfur cluster and only signals soxS when the iron-sulfur cluster is in its oxidized $[2Fe\text{-}2S]^{+2}$ state [46]. A common motif in these types of processes is that a reporter protein needs to be oxidized to affect transcriptional processes and often involves a thiol or metal complex. The best characterized adaptive pathway to oxidants and electrophiles in mammalian systems is the nuclear factor E2-related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathway [47, 48]. Nrf2 is responsible for both basal and adaptive antioxidant levels in response to oxidative stress. Nrf2 levels are stabilized during oxidative or electrophile stress by altering the binding of Kelch like-ECH-associated protein 1 (Keap1) and Cullin 3 which degrades Nrf2 by the ubiquination pathway [49, 50]. Nrf2 then binds ARE elements in the upstream promoter regions of many genes associated with antioxidant activity and xenobiotic metabolism [47, 51]. Another similar cell signaling pathway that is commonly affected by O_2 is the hypoxia-inducible factor (HIF) which under normoxic conditions is rapidly turned over by ubiquination but is stabilized during hypoxia [52, 53]. It is interesting to note that many of the electron transport systems often consume O_2 and can cause local hypoxia and may indirectly activate HIF signaling. Conversely, antioxidant dismutation reactions produce O_2 that may suppress HIF signaling [54, 55]. A rapidly growing field suggests that oxygen derived reactive species can directly or indirectly affect kinase signaling pathways [56–58]. There have been a number of kinase and phosphatase activities shown to be modulated by oxygen derived reactive species [59, 60]. These kinase pathways control a number of critical cellular functions from cell division and metabolism to cell death. A general theme of these reactions involves critical cysteine residues that are selectively oxidized by peroxide or electrophiles formed during oxidative events such as lipid peroxidation [61, 62]. An example of this is 4-hydroxy-2-nonenal modification of SHP-1 [63]. Another indirect process is the peroxide-mediated formation of an intermediate sulfenic acid that undergoes a glutathionylation reaction as has been shown for PTP1B and PTEN [64, 65]. These types of processes underscore how pharmacologic levels of

antioxidants may disrupt these signaling pathways and the difficulty of assigning a specific mechanism of action.

Classic Mechanism of Action for Antioxidant Therapeutics

The classical drug development approach is to model the agent based on endogenous antioxidants and to compare the agents ability to scavenge the oxygen derived reactive species (Figure 3). Traditionally, antioxidant therapeutics have been identified from either screens for their ability to scavenge specific oxygen derived species or a more general approach at inhibiting the formation of oxidized cellular macromolecules in a system where oxygen derived species are generated [66–72]. There are advantages and disadvantages to both approaches. The main advantage of the first approach is one can characterize specific oxygen-derived reactive species that can be targeted by the therapeutic and define the rates of reaction in simple buffer systems. Most of the catalytic antioxidants developed to date have been characterized in this manner. Some groups have used this data to denounce other competitor compounds as being non-specific, inferior, or producing their effects by artifacts [73–76], when in reality their approach and compounds suffer the same issues. The major problem with this approach is that it assumes these specific targets are going to be important in the ultimate disease indication and assumes the compound must work only by directly reacting with the specific oxygen derived species at high second order rate constants. This approach also assumes that there will be no other competing reactions or side reactions that will affect the rates of reaction and specificity for the targeted oxygen derived reactive species in more complex biological systems. To my knowledge none of these assumptions have ever been validated in complex biological systems or disease models. The more general approach does not make an assumption on how the compound may be producing its antioxidant effect in the biological system. The advantage of this approach is that a relevant biological antioxidant effect will be achieved and information on the concentration of the agent necessary for the effect. The disadvantage is that one will not have detailed information on the mechanism of action and the effect may be tissue or system specific. In reality, the first approach relies on unproven assumptions and often lacks a proven biological mechanism of action, thus is not much different than the second approach but leads to misconceptions in the field. The next section will start to probe Pandora's box of not so classy but potentially more biologically relevant antioxidant mechanisms of actions.

Pandora's Box of Potential Antioxidant Mechanism(s) of Action

The most popular and general mechanism of action by which antioxidant therapeutics work is that they protect cellular macromolecules from oxidation that leads to dysfunction [77]. This mechanism is probably most appropriate for injury and disease due to large increases in oxidant exposures that occur during industrial accidents involving oxidizing agents such as chlorine [78] and phosgene gas [79], occupational exposures to metals [80–82], ambient pollution exposures [83–85], cigarette smoke exposures [86–88] and radiation exposure [89, 90]. Under these scenarios, the endogenous antioxidant and repair defenses are overwhelmed causing injury and cell death. However, recent data has suggested that antioxidant agents may be better at affecting the delayed response of endogenous oxygen derived reactive species in response to these types of challenges on the less affected surviving tissue [18, 91–94]. A striking finding in radiation-mediating tumor killing studies was that the antioxidant treatment did not spare the tumor from radiation-induced cell death which implies the antioxidants did not scavenge the initial radiation-induced oxygen derived species. Given the earlier discussion on the numerous ways the body can endogenously produce oxygen-derived reactive species through alterations in electron transport systems one can envision numerous mechanisms that an agent could attenuate these responses without actually directly scavenging oxygen–derived reactive species. For example, the

NOX electron transport system often requires proper assemble of its components in order to generate O_2^- [95]. There are a number of agents that block this process and in doing so produce similar effects as if an agent directly scavenged O_2^- [96]. Additionally, there are a number of agents that can utilize the electrons transferred from NADPH to flavin subunits and use them to scavenge O_2^- [72, 97]. There are agents that block the transfer of electrons from NAD(P)H to the flavin subunit [96]. The biological outcomes of these separate mechanisms of action are exactly the same and could be falsely interpreted as a SOD mimic effect. Lastly, there is evidence that some managanese porphyrins can redox cycle with tetrahydrobiopterin which is required by nitric oxide synthase for nitric oxide production thereby suppressing NO production [98]. All these mechanisms could decrease the burden of oxidized cellular macromolecules. The redox potential of metal containing antioxidants is often touted as proof of optimizing a compound's dismutation activity [74]. However, some of these mechanisms associated with electron transport systems might correlate to a compound's redox potential but have nothing to do with a dismutation reaction. Now apply this concept to the hundreds of different electron transport systems in organisms and one gets a glimpse of the magnitude of Pandora's box.

Going back to the concept that organisms have co-evolved with oxygen raises the possibility that some proposed antioxidant agents may actually be weak pro-oxidants and activate biological sensors to evoke a coordinated antioxidant adaptive response. A major sensor for oxidative and electrophilic stress is the nrf2/ARE system [99]. Many natural products are thought to produce their antioxidant effects by activating the nrf2 system. Flavones and chalcone have been shown to induce the nrf2 system by increasing endogenous antioxidant expression [100, 101]. Redox active metal containing compounds are readily reduced in biological systems and can redox cycle with oxygen to paradoxically generate oxygen-derived reactive species [102]. However, they can also scavenge these reactive species so the net effect will be a decrease in their scavenging ability and this will be amplified under condition of higher oxygen tensions. Some groups claim that the so called superoxide specific mimics, such as the macrocyclic agents, are specific because they can only perform one electron transfers [73]. However, it is equally likely that macrocyclic agents can back cycle to produce superoxide under reducing conditions found in biological systems. These types of reactions could lead to paradoxical superoxide production and nrf2 induction. However, it is likely not all compounds will do this equally so this may be more likely for some antioxidant agents than others. For instance, a manganese porphyrin has been shown to not induce nrf2 responsive gene products such as HO-1 and GCL directly in cell culture and actually blocked chalcone-mediated induction of these two gene products [101]. These finding may suggest some antioxidant agents may actual block adaptive responses to oxidative stress and may work best when given after oxidant exposures.

Oxidative stress has been shown to induce HIF signaling and many antioxidants have been shown to attenuate oxidant-induced HIF signaling [103]. HIF signaling components are rapidly degraded under normal oxygen tension due to the requirement of oxygen by prolyl hydroxylases [104]. Prolyl hydroxylases are iron containing dioxygenases and it is interesting to note than iron and manganese porphyrins are often used to model enzymatic hydroxylation reactions [105, 106]. One could speculate that manganese or iron prophyrins could inhibit HIF signaling by directly hydroxylating HIFs. A consequence of dismutation of superoxide and hydrogen peroxide is the generation of oxygen which is known to suppress HIF signaling. SOD and catalase mimics are thought to suppress HIF signaling by scavenging oxygen-derived reactive species but could also be producing biological effects indirectly by suppressing HIF signaling through local increases in oxygen tension. Recent studies with manganese porphyrins have shown suppression of radiation-induced HIF signaling and assumed the mechanism to be through scavenging oxygen-derived reactive species [107–109]. Teasing out the actual mechanism(s) rather than assuming the

mechanism based on artificially defined antioxidant activities are more challenging and rarely done.

Redox signaling often involves oxidative modification of cysteine residues that can produce a wide variety of effects that can be beneficial or detrimental during oxidative stress. There are numerous redox active cysteine residues on proteins that upon oxidative modification change the protein's activity. Some of these effects are direct but many may be indirect such as changing the ratio of reduced to oxidized GSH ratios. A number of higher oxidation state of the cysteine thiol have been found under conditions of oxidative stress including disulfide (RS-SR), sulfenic acid (RSOH), sulfinic acid (RSO_2H), and sulfonic acid (RSO_3H). Each increasing oxidation state of the thiol is less reversible and less susceptible to antioxidant rescue [110]. A number of studies have shown that some of the antioxidants can attenuate the accumulation of protein thiol oxidation [21, 22, 111]. However, the exact mechanism(s) by which this is achieved is rarely reported.

In summary, there are a large number of potential mechanisms by which antioxidant therapeutic agents can produce protective effects in models of oxidative stress beside the often assumed ability to directly scavenge oxygen-derived reactive species and some groups are starting to acknowledge and explore this reality [112–114]. Many of the mechanisms involve potential interactions with NAD(P)H oxidoreductase electron transport systems such as those listed in table 1. If these NAD(P)H oxidoreductase systems are potentials sources for oxygen-derived reactive species then the interaction will "mimic" an antioxidant effect. Conversely, if these NAD(P)H oxidoreductases are predominantly antioxidant systems then their inhibition may lead to a pro-oxidant effect and evoke endogenous antioxidant responses. Likewise redox cycling with these oxidoreductases with oxygen may also evoke local changes in oxygen tensions that will affect the HIF signaling pathways, potential activation of the nrf2 signaling pathway, and changes in thiol redox balance. All these potential confounding pathways unleashes the full potential of Pandora's box and often makes it very difficult to fully define the critical mechanism(s) of action by which antioxidant therapeutics produce their beneficial effects in complex biological systems. However the current practice of assuming the mechanism of action of antioxidant therapeutics is due to their chemical reactivity in more simple chemical systems is often deceptive and misleading. It is very likely that each antioxidant agent will have different mechanism(s) of action and that this may change with model and disease state. Welcome to Pandora.

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Highlights

- During evolution, biological systems have adapted to the abnormal biology of O_2.
- Cells use O_2's reactive nature in many different electron transport systems.
- Disruption of electron transport systems can alter oxidant production.
- The numbers of different mechanisms of action for antioxidants are numerous.
- Reliance solely on chemical reactivity of an antioxidant can be misleading.

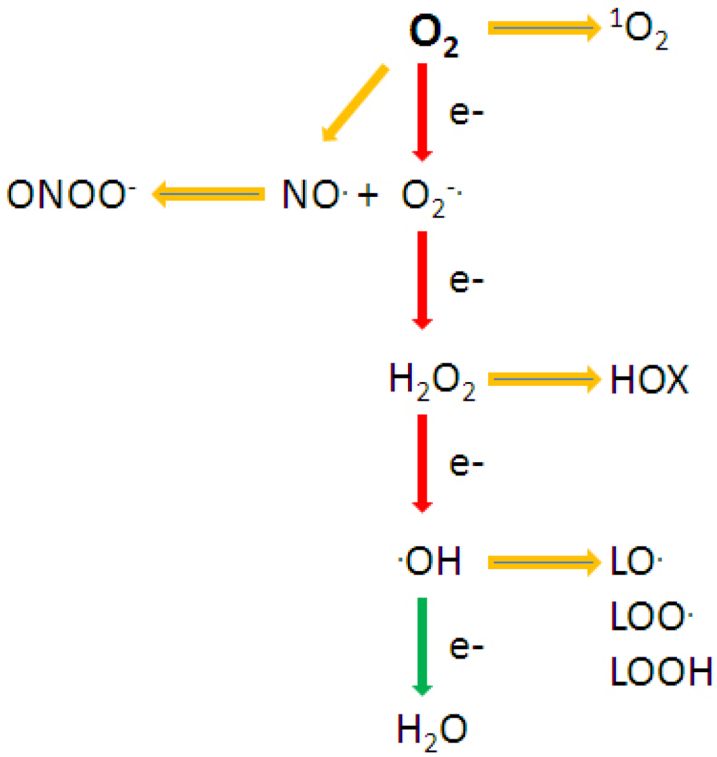

Figure 1.
The partial reduction of dioxygen (O_2) and the formation of a number of oxygen-derived reactive species: singlet oxygen ($^1O_2$), superoxide ($O_2^{-\cdot}$), nitric oxide ($NO^{\cdot}$), peroxynitrite ($ONOO^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), lipid alkoxyl radical ($LO^{\cdot}$), lipid peroxyl radical ($LOO^{\cdot}$), and lipid peroxide (LOOH).

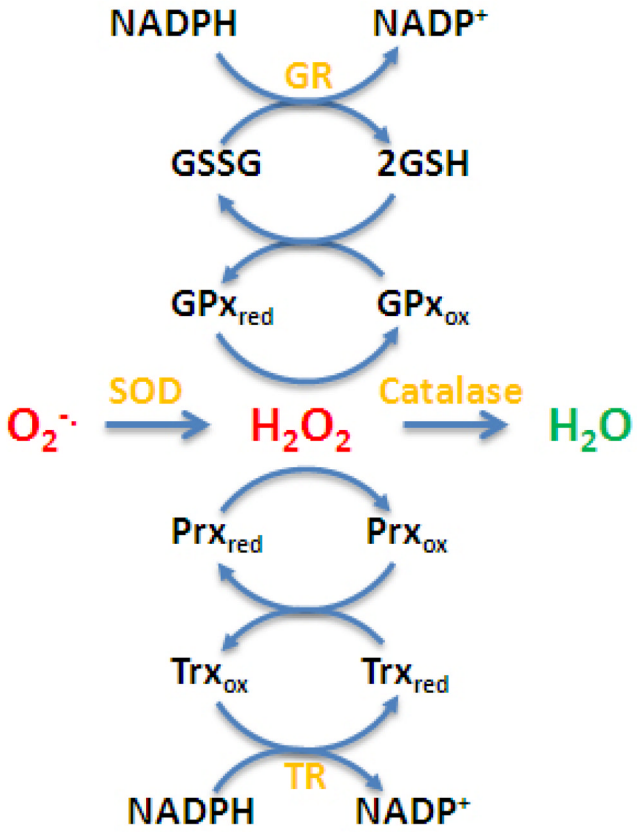

Figure 2.
The major endogenous antioxidant pathways that scavenge oxygen-derived reactive species. Superoxide ($O_2^{-\cdot}$) is scavenged by superoxide dismutase (SOD) and the hydrogen peroxide (H2O2) product can be scavenged by catalase or two thiol based systems. The glutathione (GSH) system utilizes glutathione peroxidase (GPx) and GSH. The glutathione disulfide is recycled by glutathione reductase (GR) that utilizes NADPH. The thioredoxin (Trx) system utilizes peroxiredoxins (Prx) that are recycled by thioredoxin (Trx). The oxidized Trx is recycled by thioredxin reductase (TR) that utilizes NADPH.

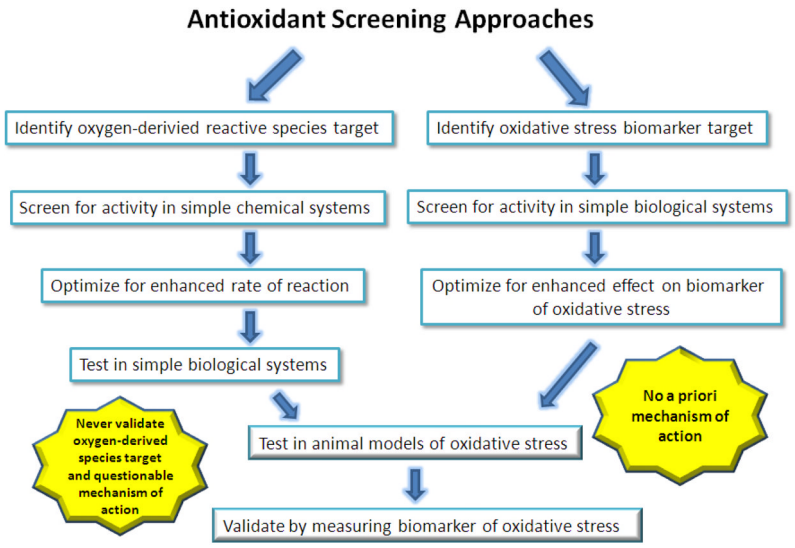

Figure 3.
Current concepts and concerns about screening strategies for antioxidant therapeutics.

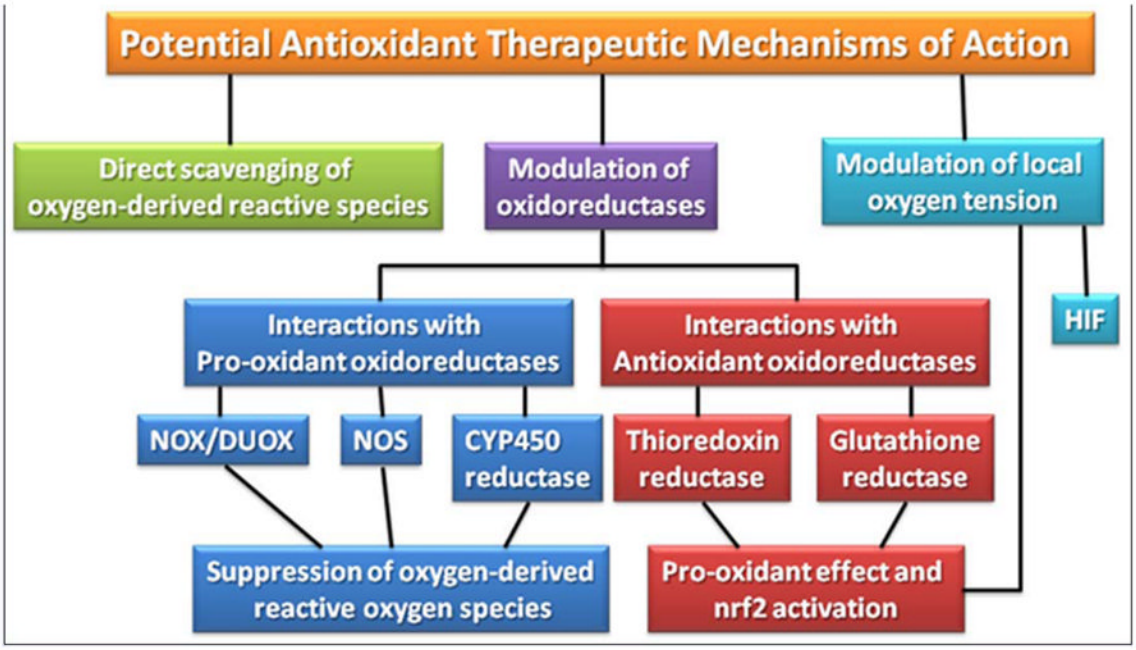

Figure 4.
Potential mechanisms of action for antioxidant therapeutics.

Table 1

Potential NAD(P)H Dependent Electron Transport Systems for redox reaction with Antioxidants

Biological Electron Transport Systems	Category
NOX/DOUX	Pro-oxidant
Xanthine oxidoreductase	Pro-oxidant
Cytochrome b5 reductase	Pro-oxidant
P450/P450 reductase	Pro-oxidant
Nitric oxide synthase	Pro-oxidant
Ubiquinone oxidoreductase	Pro-oxidant
Heme oxygenase	Antioxidant
Biliverdin reductase	Antioxidant
Quinone oxidoreductase (NQO1)	Antioxidant
Thioredoxin reductase	Antioxidant
Glutathione reductase	Antioxidant
Monodehydroascorbate reductase	Antioxidant