Antioxidant defenses in human blood plasma and extra-cellular fluids[1]

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Abstract

I had the fortune to be introduced to Helmut Sies during the mid 1980s, while working as a post-doctoral scientist at the University of California, Berkeley. At that time, Helmut was a frequent visitor of the Bruce Ames' laboratory and a leading authority in antioxidants and oxidative stress. His concepts, ideas and willingness to listen and make constructive suggestions have been far-reaching and visionary. Moreover, they have also been highly infectious, so much so that much of my research to this day has been on the same topic. The following is a personal recount on how the field of antioxidants has evolved since those exciting days in Berkeley.

Keywords

Antioxidants, vitamin E, ascorbic acid, pro-oxidant, heme oxygenase-1

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[1]Dedicated to Helmut Sies for his visionary contributions to the field of oxidative stress and antioxidants. "Vision is the art of seeing things invisible" — Jonathan Swift

Introduction

My association with Helmut Sies began nearly 30 years ago, while working as a post-doctoral scientist in the laboratory of Bruce Ames at the University of California, Berkeley. It was a stimulating time! Having developed the Ames test for chemical mutagens in the 1970s, Ames realized during the 1980s that the human diet contains many mutagens and carcinogens. To allow the ranking of the relative hazards of human exposure to known natural and synthetic carcinogens, Ames developed an index that expressed the human potency of a carcinogen as a percentage of its potency to laboratory rats and mice. Together with Lois Gold, Ames published a series of provocative papers, such as "Pesticides, 99.99% all natural".[1] They pointed out that, except for some occupational exposures (*e.g.*, medical, asbestos, pesticide), human exposures to synthetic (man-made) carcinogens are generally at low doses, and that "humans ingest about 10,000 times more natural pesticides, by weight, than synthetic pesticides". Not surprisingly, Ames came into conflict with environmental groups that, at the time, were waging campaigns against the use of certain synthetic chemicals.

At the same time, there was an increasing awareness that many mutagens and carcinogens may act through the formation of reactive oxygen species, giving rise to DNA damage and mutations.[2] This realization provided a context for Helmut to be a frequent visitor of the Ames' laboratory, and for young post-docs like myself to become exposed to some of Helmut's views of and ideas on 'oxidative stress'. Indeed, Helmut had a profound impact on my research in the areas of antioxidants and oxidative stress. At that time Helmut defined the term 'oxidative stress' as "a disturbance in the prooxidant – antioxidant balance in favor of the former".[3] Helmut pointed out that oxygen free radicals were considered synonymously with "reactive" or "aggressive" species that potentially cause oxidative damage, essentially as the price for the favorable aspects of aerobic life.[3]

As a corollary to Helmut's concept of oxidative stress, the research interest in "active oxygen" extended to the role of antioxidants in the protection against oxidative damage. Many laboratories made important contributions to this area, with Helmut taking a leading role[4,5] jointly with others, including Barry Halliwell[6] and Bruce Ames.[7] Much of this research focused on cellular enzymatic antioxidant defenses. For example, work from Peter Cerutti's laboratory demonstrated the importance of superoxide dismutase and catalase as regulators of the tumor promoting activities of reactive oxygen species.[8] By comparison, my interests were directed more towards the antioxidant defenses in the extracellular space, not least because work by Barry Halliwell and his colleagues highlighted the compositional difference in the make-up between cellular and extracellular antioxidant defenses.[9]

The changing view of extracellular 'antioxidants'

Most commonly, antioxidant defenses in extracellular fluids were studied in human plasma. Our early views on the molecular action of extracellular (as well as cellular) antioxidants was simplistic: reactive oxygen species cause damage and antioxidants attenuate this damage by scavenging the reactive species, decrease the source of reactive species and/or repair biomolecules that had been 'damaged' or become 'oxidized'. However, this view has evolved and changed substantially as our knowledge in this area of research has increased, as illustrated in the following.

Early considerations

Early studies on antioxidants focused on the important principles of thermodynamics and kinetics, with biological systems or models thereof most commonly examined in isolation and as a 'homogeneous' system. This is perhaps best illustrated by studies addressing antioxidants in the lipid phase of human plasma. To accurately study the kinetic properties of lipid-soluble antioxidants, chemists including Keith Ingold, introduced the controlled formation of reactive oxygen species, *e.g.*, by thermolabile azo-initiators, in conjunction with accurate quantification of the oxidative damage, *e.g.*, as assessed by the extent of oxygen consumption.[10] This approach yielded important information. For example, it established that kinetically, α-tocopherol is the most effective peroxyl radical scavenger of the vitamin E family of compounds and that, *quantitatively*, vitamin E is the major antioxidant in human plasma and red blood cells.[11] It also clarified that β-carotene and other carotenoids are not major antioxidants in human plasma in quantitative terms.[12] Being able to *quantify* how effectively an antioxidant 'trapped' peroxyl radicals, also allowed Ingold and co-workers to assess the relative contribution of each plasma antioxidant to the total peroxyl-radical trapping antioxidant activity.[13] The principle has since been exploited commercially by assays such as the TRAP (total radical-trapping parameter) assay.

In addition to these kinetic considerations, early studies also focused on thermodynamic properties of free radicals and their use to predict a pecking order, or hierarchy, of free radicals and antioxidants.[14] Applying these principles led Garry Buettner to conclude that vitamin E is "the primary lipid-soluble small molecule antioxidant and vitamin C the terminal water-soluble small molecule antioxidant".[14] By highlighting the unique "interfacial" nature of the reaction of the α-tocopheroxyl radical in the lipid phase with ascorbate in the water-phase, the thermodynamic principles applied by Buettner supported two separate concepts of antioxidant activities: compartmentalization (*vide infra*) and interaction between vitamin E and ascorbate.

The first direct evidence for the interaction between vitamin E and ascorbate was reported by John Packer, Trevor Slater and Robin Willson at Brunel University in 1979.[15] Subsequent work by several laboratories including that of Lester Packer at UC Berkeley extended the notion that small molecule antioxidants do not work in isolation but rather as 'antioxidant network'. This 'network' not

only includes additional lipid-soluble (*e.g.*, ubiquinols[16] and bilirubin[17]) and water-soluble small molecules (*e.g.*, lipoic acid[18]), but also protein-bound antioxidants (*e.g.*, albumin-bound bilirubin[17]) and enzyme, such as succinate-ubiquinone reductase[19] and cytochrome *c*.[20]

Refining the concepts of antioxidant activities

While the early studies referred to above provided valuable data on the chemical reactivity of antioxidants, the biological relevance of the information obtained was limited in a number of ways. Foremost, the quantitative consideration of antioxidant efficacy and contribution to total radical-trapping antioxidant activity is based on 'closed' test systems, where the individual or combined radical trapping activity of antioxidant(s) is determined *in vitro* and in isolation from their biological environment. In contrast, biological systems are 'open', *i.e.*, they allow for the continuous replenishment of at least some of the antioxidants. Moreover, it is important to consider the possibility that the rate of antioxidant replenishment varies under different conditions, and in fact may increase under conditions of oxidative stress as a result of induction of antioxidant defense systems. As a result, *qualitative* characteristics have been considered as important if not more important than quantitative aspects of antioxidant efficacy. This has led to the concept of 'first-line' antioxidants, defined as antioxidants that (i) are consumed first when a biological system is exposed to reactive oxygen species; and (ii) prevent oxidative damage while present. Accordingly, ascorbate[21] and ubiquinol-10[22] were identified as the first-line antioxidants in the aqueous and lipid phase of human plasma, respectively, with Balz Frei playing a leading role in this aspect of antioxidant research.

A second important refinement of our concepts of antioxidant activity and efficacy relates to the spatial compartmentalization of free radical/redox reactions with antioxidants. This notion, now well established to be important in cellular homeostasis and responses,[23] also applies to human plasma. This is illustrated strikingly by the principles of "tocopherol-mediated peroxidation" of lipids in low-density lipoprotein (LDL), we discovered in 1992.[24,25] Here, it is the physical entrapment of α-tocopherol in the LDL particles and their spatial compartmentalization from the surrounding aqueous environment that dramatically alter the activity of the vitamin from an efficient chain-breaking antioxidant (in homogeneous systems) to a potential lipid peroxidation chain-carrying pro-oxidant. Physical compartmentalization of antioxidant activity in human plasma is not limited to LDL, but extends to other lipoproteins as well as plasma proteins. An example of the latter is albumin with its high binding affinity for the antioxidant bilirubin. Thus, albumin-bound bilirubin effectively inhibits radical-induced chain oxidation of albumin,[26] just as it can transfer reducing equivalents to α-tocopherol in LDL.[17]

The discovery of tocopherol-mediated peroxidation led to two additional refinements of our concepts of antioxidant activities. Firstly, it led to the realization that the 'switch' for α-tocopherol changing from an antioxidant to a pro-oxidant in LDL is determined by both, the type of radicals

involved and the rate at which radicals are formed and hence react with the vitamin.[27] Moreover, this 'switch point' varies with the size of the lipoprotein particle, with the likelihood for a pro-oxidant activity of vitamin E increasing with increasing surface-to-volume ratio of the lipoprotein particle.[28] These findings demonstrate that the biological efficacy of an antioxidant is not determined exclusively by its chemical and physical properties, but can also be affected by the oxidizing environment in which it acts. Secondly, the principles of tocopherol-mediated peroxidation led to the concept of co-antioxidation. Co-antioxidants are defined as compounds that reduce α-tocopheroxyl radical *and* accelerate the diffusion of radicals between the (lipoprotein) lipid and aqueous phase.[29] This definition of co-antioxidation represents an extension of the concept of 'antioxidant network' (*vide supra*) to a large number of chemicals, including many natural and synthetic compounds.[30]

A final example of the advancing concepts of antioxidant activities in human plasma comes from work carried out by Helmut Sies while on sabbatical in my laboratory in Sydney in 1992. At that time one of Helmut's many interests centered on ebselen, a glutathione peroxidase mimetic that requires the presence of low-molecular-mass thiols to be active. In circulation ebselen is transported as an albumin complex that was thought to be inactive. Despite this, however, we demonstrated that albumin-bound ebselen added to whole blood was able to chemically reduce LDL-associated lipid hydroperoxides.[31] Interestingly, such reduction was not observed when albumin-bound ebselen was added to plasma, *i.e.*, in the absence of blood cells.[31] These results suggested that ebselen may be considered as a drug for extracellular targets, but also that blood cells can impact on the activity of low molecular mass plasma antioxidants.

'Antioxidants' as inducers of heme oxygenase-1

With the advent of co-antioxidants and our ability to prevent LDL oxidation,[30] research in my laboratory in the mid to late 1990s focused largely on testing the causal relationship between LDL lipid oxidation in the arterial wall and atherosclerosis. We employed several animal models of atherosclerosis and related diseases in conjunction with pharmacological means to modulate LDL oxidation *in vivo*. We observed co-antioxidants of LDL's α-tocopherol to consistently and effectively inhibit arterial lipoprotein lipid oxidation.[32,33] However, inhibition of lipoprotein oxidation in the arterial wall did not consistently translate into inhibition of atherosclerosis. In fact, in some case, arterial lipoprotein lipid oxidation inversely related to the disease outcome.[34] We reconciled these findings, together with other studies, with arterial LDL lipid oxidation representing a byproduct of, rather than the cause of, atherosclerosis (see Ref.[35] for a definitive review).

The observed ability to dissociate arterial lipoprotein lipid oxidation from vascular disease raised a number of new questions on the role of oxidative stress and antioxidants in atherosclerosis (reviewed in Ref.[36]). Among these, we focused our research on the question of how certain antioxidants protect against atherosclerosis if not via inhibition of LDL oxidation. In doing so, we

chose the antioxidant probucol, because this phenolic compound is effective in humans for the treatment of hyperlipidemia. Moreover, earlier studies by Daniel Steinberg and colleagues reported probucol to inhibit experimental atherosclerosis independent of its lipid-lowering activity, and this was presented as one of the pillars of the original Oxidative Modification Hypothesis of atherosclerosis.[37] Our studies discovered that probucol inhibits experimental vascular disease via up-regulation of heme oxygenase-1 (Hmox1) rather than via inhibition of LDL oxidation.[38-40] Hmox1 is a stress protein that is induced by a variety of stressors, including oxidants, and that protects cells against a number of insults, including oxidative stress.[41,42] Additional mechanistic studies unexpectedly revealed that vascular protection by probucol was associated with oxidation of the drug[34] and required its sulphur atoms, whereas the phenol moieties of probucol were insufficient for protection.[43] These findings indicate 2-electron rather than radical oxidants as important contributors to atherogenesis, and that a product derived from probucol via 2-electron oxidation was likely responsible for induction of the protective Hmox1. More recently, certain polyphenolic antioxidants, in particular quercetin and theaflavin, were also reported to attenuate experimental atherosclerosis by inducing heme oxygenase-1 and increasing nitric oxide bioavailability.[44] This suggests that an indirect mode of action, *i.e.*, the induction of protective enzymes, may be a common path by which certain antioxidants provide protection *in vivo*. We are currently investigating whether, similar to the situation with probucol, prior oxidation is required for polyphenols to be able to induce Hmox1. Preliminary data suggest that this is indeed the case, at least for quercetin (unpublished observations). If so, this raises the possibility that in some circumstances, 'antioxidants' may provide biological benefit by acting as oxidants or oxidant stressors.[41] With other words, the advantage provided by the antioxidant may not rest with it scavenging reactive oxygen species and preventing oxidative damage directly, but rather by the ability of its oxidized form to evoke an adaptive response that then provides protection independent of the antioxidant.

Antioxidants as electrophiles

Most recently, Forman and colleagues presented an exciting new concept of how antioxidants in fruits and vegetables may exert their health-protective effects.[45] They proposed that enzymatic removal of non-radical electrophiles, *e.g.*, hydroperoxides, in two-electron redox reactions rather than free radical scavenging as a major antioxidant mechanism. Moreover, they suggested oxidative activation of the NF-E2-related factor 2 (Nrf2) signaling pathway as a major mechanism of action of nutritional antioxidants. The underlying concept of this proposal is consistent with our observations that it is the oxidized forms of probucol and quercetin rather than their respective antioxidant-active reduced forms that provide biological protection (*vide supra*). As Hmox1 is a well-known downstream target of Nrf2,[41] induction of Hmox1 by probucol and quercetin could conceivably be preceded by up-regulation of Nrf2.

The proposal by Forman and colleagues has clear merit and likely extends to synthetic antioxidants. For example, and as implied above, ebselen has electrophilic properties, as it targets protein cysteine residues for oxidation. Indeed, several groups reported ebselen to also modify cysteine residues in Kelch-like ECH-associated protein 1 (Keap1), the electrophile sensor protein that represses the ability of (Nrf2) to induce phase 2 detoxification response.[46,47] Similar to ebselen, the synthetic antioxidant *tert*-butyl hydroquinone is also induces the Nrf2 response,[47] and this may extend to other phenolic antioxidants.[48] Thus, some antioxidants may paradoxically provide biological protection against oxidative stress by acting as oxidants.

Conclusions

Our understanding of small molecule antioxidants has changed dramatically over the last three decades. We now recognize that *in vivo* these antioxidants likely act in concert with each other as well as proteins and enzymes, and antioxidants are expected to engage in redox reactions within discrete 'compartments'. Identifying and being able to study these compartments in a biologically meaningful way is one of the future challenges in this area of research. To better comprehend the *in vivo* action of antioxidants, we also must obtain a better understanding and develop better tools for the quantification of the rate at which different reactive oxygen species are formed *in vivo*, as well as the time-dependent changes (consumption and replenishment) of antioxidants within the compartment(s) of interest. Finally, in light of the fact that antioxidants provide benefit neither generally nor consistently, we must consider that benefit by 'antioxidants' – where observed – is achieved indirectly via modulation of biological processes such as an increase in protective metabolic and/or signaling pathways, rather than via direct inhibition of an oxidative process thought to be involved in the pathogenesis studied. To bring this story full circle, it is with Helmut's vision and multiple contributions, that this area of research has made so many substantial advances.

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