Minireview

Free radical biology – terminology and critical thinking

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Abstract What is an antioxidant? Can one, at a cellular level, speak of direct and indirect antioxidants? Can oxidative stress be quantified and characterized? What are the oxidant species that may have regulatory functions in a cell? Since the above concepts have become of frequent use in all Journals it may be appropriate if some critical thinking outlined in this review could become available to a broad public.
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1. What is an antioxidant?

A generic definition of an antioxidant is not experimentally constructive unless it is associated with the notion of the oxidant that has to be neutralized. Furthermore, the concept of an antioxidant in vitro should not be extended to cells, to organs, to animals or to populations until the evidence has been obtained. Moreover, a molecule demonstrated to have antioxidant properties in vitro might have *additional* properties in a more complex system. For example, estrogens have been implied as antioxidants through lipoprotein and neural protection [1,2]. However, estrogens act mainly through receptor-mediated signalling and not by the weak antioxidant properties of the molecule. Similarly, retinol, an antioxidant in vitro, acts in its association with opsin, to produce a reversible complex, rhodopsin, whose interaction with light initiates the process of vision. It is well established that this event is due to isomerization of the pigment and not to a redox or antioxidant process. Unfortunately however, confusion about this issue exists as a number of studies have classified retinol as a plasma antioxidant and have studied its relationship with diseases [3–6]. Similarly, melatonin has been considered an antioxidant [7–11] but at the concentrations available in the human body it is unlikely to have such an effect. The use of 2-[^{125}I]iodomelatonin has allowed the exact localization and characterization of high-affinity melatonin receptors that signal through the $G_{i/o}$ class of G proteins. Molecular cloning of melatonin receptor genes has confirmed that most, if not all, high-affinity melatonin binding sites represent the G protein-coupled melatonin receptors [12]. The hormonal action of melatonin generating the circadian rhythm in humans is unrelated to its antioxidant properties. In a similar vein, are the natural products called phytoestrogens responsible for their effects in humans because of their antioxidant properties or because of their estrogen mimicry [13–15]? Lipid radical chain-breaking properties have been ascribed to α-tocopherol but recently, new, non-antioxidant effects have come to light for this important micronutrient [16–19]. In conclusion, we suggest that calling a molecule an antioxidant at a cellular level or in vivo simply because it has chemical antioxidant properties should not be encouraged.

2. Are antioxidants identified in in vitro tests bioavailable?

Another problem that in vitro antioxidants encounter is their bioavailability [20]. For example, perfusion of isolated small intestine with procyanidin dimers extracted from cocoa indicated that they are transferred to the serosal side of enterocytes to only a very small extent. Moreover, the transferred form appears to be *O*-methylated and not to possess antioxidant activity. Importantly, the cell protection exerted by the *O*-methylated or by the non-methylated forms are very similar indicating that protection is not linked with a direct antioxidant event [21]. Bioavailability is also an issue when simple attempts are made at extrapolating in vitro to in vivo situations [22,23]. One of the most studied 'antioxidants' is α-tocopherol and problems still arise regarding the competition of different forms [24–26], their uptake and the synergistic or the inhibitory role of other compounds. For compounds like polyphenols much has to be done before we can believe they are effectively transported, and that they reach cells in an 'antioxidant' form through which they exert positive effects at a cellular and an organism level [27–30]. Indeed, much still has to be learnt about the uptake, biotransformation, and tissue distribution of molecules regularly thought of as 'antioxidants' before we can truly indicate that they have such function in vivo.

3. At a cellular level, can one speak of direct and indirect antioxidants?

If a molecule has the ability of inducing gene expression (for example via the antioxidant response element (ARE) present in the promoter region of a number of phase 2 detoxifying enzymes) can this molecule be called an antioxidant? Not automatically and without a more detailed knowledge of

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the involved pathways. In fact ARE and the transcription factor Nrf2 are essential for inducible and/or constitutive expression of a group of detoxification and antioxidant enzymes [31]. They mediate gene regulation by oxidative stress as well as by electrophiles and so-called antioxidants [32]. Or, given that α-tocopherol inhibits NADPH oxidase in macrophages (and thus the oxygen burst) by preventing phosphorylation of P47 and assembly of the active oxidase [33], can it be considered an indirect antioxidant? If we take this line, then staurosporine is also an antioxidant, as it is a known inhibitor of protein kinase C and of the related P47 phosphorylation. Conversely, antimycin A (with no reactive oxygen groups) blocks the electron flow in the mitochondrial complex III, and induces superoxide and hydrogen peroxide release [34,35]. Is antimycin A therefore a pro-oxidant? On the other hand, tyrphostin, a selective epidermal growth factor receptor kinase inhibitor, suppresses the hydrogen peroxide-induced increase in aldose reductase mRNA and enzyme activity [36]. Is tyrphostin therefore an antioxidant? By raising these examples we aim to convince that the definition of a pro-oxidant or antioxidant which is remotely linked with reactive oxygen species production or elimination is often not helpful in understanding the mechanistic action of the compound. Fig. 1 illustrates in a diagrammatic way the fate of a bona fide, in vitro antioxidant when introduced into the human body. The events related to its absorption and modification are indicated. Through methylation or glucuronidation the antioxidant properties are lost, but still the molecules may exert effects, at a cellular level, that are similar to the original antioxidant. The figure illustrates moreover that antioxidants and modified antioxidants can equally regulate gene expression. The genes which are under the control of these ligands may or may not be involved with the metabolism of reactive oxygen species.

4. Can 'oxidative stress' [37,38] be quantified and characterized?

The theoretical concept is simple: 'Oxidative stress' is defined as *an imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage* [39,40]. Non-cyclooxygenase-derived prostanoids, transient enhancement of heme oxygenase 1, ascorbate free radical, salicylate, glutathione antioxidant system, advanced oxidation protein products, ubiquinol/ubiquinone ratio, oxidative DNA damage in the form of 8-hydroxy-2′-deoxyguanosine, malonyldialdehyde content of cell membranes, the plasma levels of 8-epi-prostaglandin F2α, increased 8-isoprostane and many other markers of 'oxidative stress' have given very discordant results [41–52]. Similarly antioxidant therapies aimed at producing a re-equilibration of the 'oxidative stress' have given conflicting results. Again, citing the most studied 'antioxidant' vitamin E, the conclusions as to its in vivo antioxidant efficacy are far from being unanimous [53,54]. The description of an 'oxidant stress' situation is only useful if the molecular details of the imbalance are known. The lack of such detail in many pub-

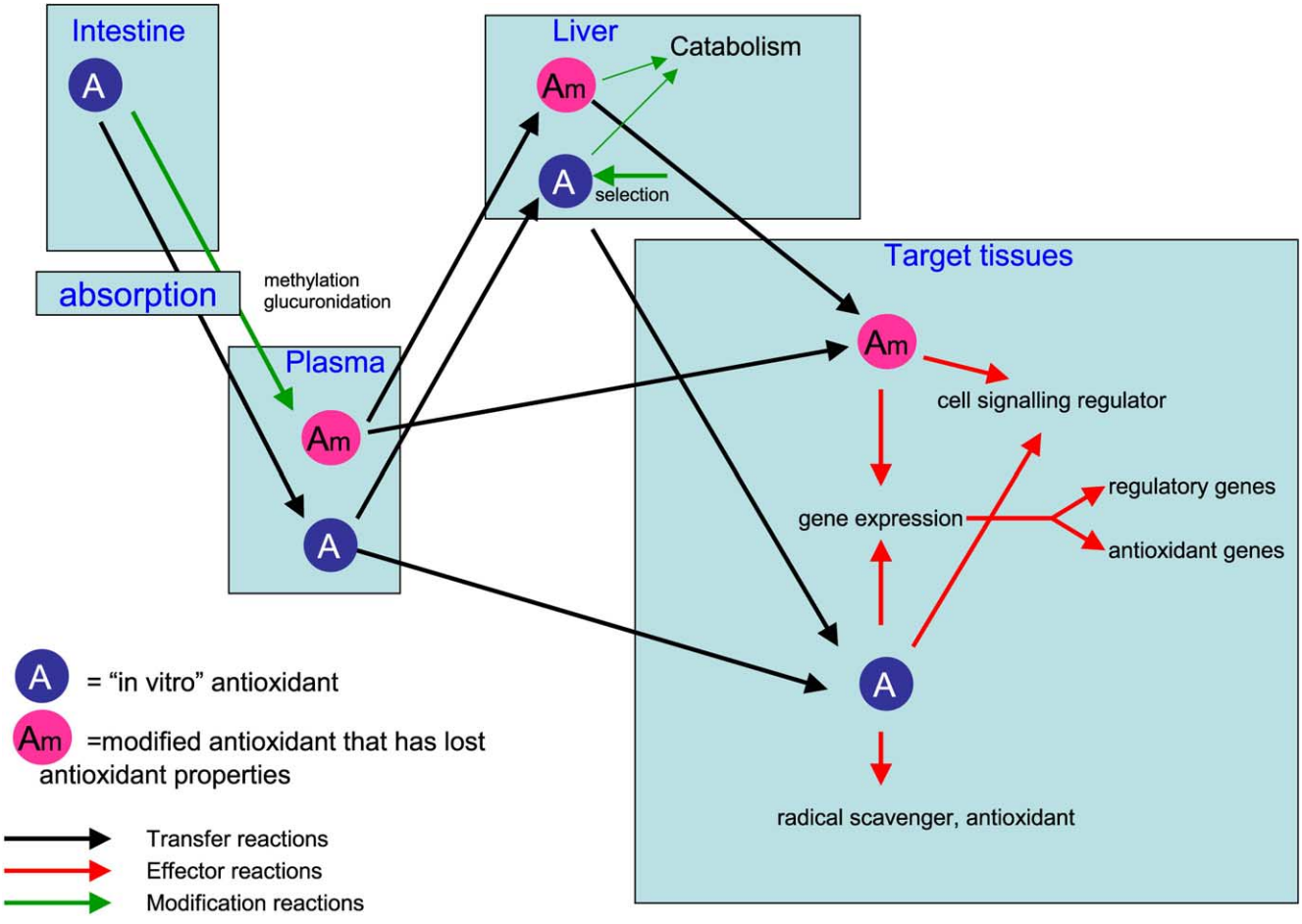

Fig. 1. Absorption, modification, distribution and effects of molecules with in vitro antioxidant properties. For explanation see text.

lished antioxidant intervention studies has compounded confusion in this area. In a similar fashion, it is still widely assumed that antioxidant administration will always provide benefit. This is naive thinking. Antioxidants can protect or increase injury depending on the situation and therefore their use should always be made with a full appreciation of the situation.

5. Are there oxidant species that may have a regulatory function?

Amongst all nitrogen and oxygen species that may act as signalling molecules, two (nitric oxide and hydrogen peroxide) appear to have the best theoretical qualities of stability and reactivity, needed for such a purpose. Nitric oxide's molecular mechanism of signalling has been studied in detail (for recent reviews see [55,56]). Increasing evidence indicates that the production of reactive oxygen species is precisely regulated and their downstream targets specific. Remarkable progress has been made in defining the specific redox-dependent targets of intracellular oxidants, as well as the innumerable pathways that employ oxidants as effectors in diverse processes from tumorigenesis to ageing [57]. Only some examples will be given of these important regulatory pathways.

Hydrogen peroxide stimulates c-Src-mediated big mitogen-activated protein kinase 1 and the MEF2C signaling pathway in PC12 cells. This reaction plays a potential role in cell survival following oxidative insult [58]. Hydrogen peroxide acts as a messenger in the growth factor-induced p70(S6k) signaling pathway [59]. In addition, hydrogen peroxide promotes calcium-dependent endothelial nitric oxide synthase activity through a coordinated change in the phosphorylation status of the enzyme mediated by Src- and ErbB receptor-dependent phosphoinositide 3′-kinase activation [60]. The better identification of hydrogen peroxide target molecules [61] and elucidation of the mechanism by which hydrogen peroxide acts in initiating signal transduction is an area of great promise for future research.

Oxidized lipids, to date, only appear to have a possible signalling function in pathological situations. For instance oxidized phospholipids are pro-inflammatory agonists promoting chronic inflammation in atherosclerosis; and they can inhibit expression of inflammatory adhesion molecules. They inhibit lipopolysaccharide (LPS)-induced, but not tumor necrosis factor-α-induced or interleukin-1β-induced NF-κB-mediated upregulation of inflammatory genes, by blocking the interaction of LPS with LPS binding protein and CD14 [62]. 4-Hydroxy-2,3-nonenal, a fatty acid oxidation product, has been reported to control aldose reductase expression [63]. Inhibition of the survival-promoting NF-κB signaling pathway by 4-hydroxy-2,3-nonenal may contribute to neuronal death under conditions in which membrane lipid peroxidation occurs [64]. A biphasic increase in activator protein 1 DNA binding activity, associated with increased mRNA levels of c-Jun, was also observed in response to 4-hydroxy-2,3-nonenal [65]. Membrane lipid peroxy radicals can hardly have signalling functions, under physiological conditions. In fact, due to their high reactivity, they would not be able to afford a specific and controlled information transfer. Consequently, the cellular regulation provided by α-tocopherol is unlikely to be the result of the modulation of lipid peroxy radicals.

6. Conclusions

It is time, after a period of flourishing research on oxidants and antioxidants, to critically reflect on the field. Speculation that many (if not all) diseases are related to radical damage needs to be supported by more secure data. The hope that antioxidants can prevent or cure a number of pathological situations also requires reconsideration. The relatively new notion that molecules with strong antioxidant activity in vitro may have 'non-antioxidant' effects in cells and tissues should stimulate, rather than discourage, important research in this field. Finally, the discrepancies in the outcome of intervention studies may be understood if, instead of considering the simple paradigm of bad oxidants and good antioxidants, scientists will start to talk about the real molecular function of such compounds in each particular situation.

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