Review

Lipid peroxidation, antioxidants and cardiovascular disease: how should we move forward?

Barry Halliwell

Department of Biochemistry, National University of Singapore, Kent Ridge Crescent, Singapore 119260, Singapore

Received 27 January 2000; accepted 28 March 2000

Keywords: Coronary disease; Free radicals

1. Introduction

It is widely agreed that increased consumption of fruits, grains and vegetables, decreased intake of saturated fats, a moderate degree of exercise and perhaps judicious consumption of red wine or other alcoholic beverages (or even tea) would improve the cardiovascular health of the populations in most developed and ‘near-developed’ countries [1–8]. Fruits, grains, teas, vegetables and red wines are rich in antioxidants (ascorbate, tocopherols, tocotrienols, flavonoids, other phenols and carotenoids are among the antioxidants found in various plants consumed by humans; reviewed in [9]), and so it is widely thought that antioxidants make an important contribution to this cardiovascular protective effect [9–14]. This assumption is logical, because there is good evidence that oxidative damage contributes to the pathology of atherosclerosis and vascular dysfunction generally, and that free radicals are involved in myocardial ischemia-reperfusion injury [9,15–20]. However, intervention trials with vitamin E that assess clinical end-points are giving a confused picture [21–23]. Indeed, foods and beverages derived from plants are chemically complex, and cardiovascular protective effects could also arise from many other components or mixtures of components present, including fibre, immunostimulatory agents, monounsaturated fatty acids, agents that modulate cholesterol synthesis, B-vitamins, folic acid, agents modulating nitric oxide production, and even the humble ethanol molecule itself [2,3,5,6,14,24–29].

One obvious way to assess the contribution of antioxidants to the cardiovascular protective effects of the above diets is to conduct intervention trials with single antioxidants, or combinations of a few antioxidants. For example the intervention trials with β-carotene convincingly demolished the concept that this carotenoid is an important anti-cancer agent in humans, at least in smokers [30]. Hence high plasma levels of β-carotene are negatively associated with cancer incidence because both are a consequence of eating a good diet; β-carotene is not the protective agent in that diet [30]. Indeed, the ‘antioxidant hypothesis’ might have predicted this: by comparison with flavonoids, tocopherols and ascorbate, β-carotene is a poor antioxidant even in vitro and probably unlikely to be any better in vivo [31]. This is further illustrated by the observation that increased vegetable and fruit consumption, and (under some circumstances) physical exercise, can decrease levels of oxidative DNA damage (a putative risk factor for cancer development) in humans and other animals, and high-fat diets can increase these levels, but β-carotene supplements have little or no effect on levels of oxidative DNA damage in the human body [31–42].

Is the situation any different for cardiovascular disease? There is little evidence that β-carotene has any direct effect on incidence of cardiovascular disease or diabetes [43–46]. Data on the relation of vitamin C intake to cardiovascular disease are conflicting, but no consensus has emerged for a cardiovascular protective effect [43,44,47], although the suggested association of low ascorbate intake with hypertension is intriguing [48,49]. Improved vascular function in some subjects has been reported after consumption of gram quantities of ascorbate [50–52], but it is uncertain as to whether antioxidant or other properties of ascorbate are responsible [53]. In relation to cardiovascular disease prevention, vitamin E is
perhaps the most controversial antioxidant, with results of supplementation trials ranging from dramatic protective actions to absence of effect [21–23,46,54,55].

2. Are we asking the correct questions?

Why this confusion? Let us step back for a moment to examine fundamentals. The starting hypothesis is that oxidative damage, especially lipid peroxidation, is a key contributor to the progression, and perhaps to the origin of, cardiovascular disease [9,15,16]. We know that diets rich in plant products, which in turn are rich in antioxidants, have a cardiovascular protective effect. Key questions must then surely be:

(1) Is the protective effect of such diets due, in whole or in part, to antioxidant mechanisms? In other words, do such diets decrease oxidative damage in vivo, especially lipid peroxidation, to an extent that could explain their protective effects?

(2) If the diets do decrease oxidative damage, which component(s) are responsible? Is it vitamin E, vitamin C, flavonoids, carotenoids etc.? Or do we need a mixture of components that act synergistically [56] or is the antioxidant effect due to previously unsuspected molecules?

(3) In carrying out an intervention trial with a putative antioxidant, have we succeeded in decreasing oxidative damage in the test subjects? If we have not, a negative result is predictable from our initial hypothesis, i.e. cardiovascular disease incidence will not drop because we have not inhibited the oxidative damage that contributes to it [57]. For example, a recent comprehensive review [58] concluded that there was little convincing evidence that vitamin C supplements had lowered levels of oxidative damage in human intervention studies. One reason may be that many such studies have been carried out on subjects who already had sufficient tissue and body fluid levels of vitamin C to achieve the maximal antioxidant effect. There is evidence from studies of oxidative DNA damage that intakes of ascorbate of 100–200 mg per day maximize its protective effects, and higher intakes may even increase oxidative DNA damage (reviewed in [59]). If the putative protective effect of ascorbate against cancer development is due to its antioxidant properties in protecting DNA against oxidative damage, supplementation carried out on populations who already had intakes of 100–200 mg per day would be expected to reveal no effect. As I have stressed recently [57], it is pointless to do large intervention trials with antioxidants in humans without preceding and accompanying studies to show that the antioxidant at the dose to be given is capable of decreasing oxidative damage significantly in the population in question. Unless this is shown, it will be impossible to interpret the trial results in relation to the initial hypothesis.

A parallel question is whether vitamins E, C and carotenoids exert cardiovascular protective effects unrelated to their antioxidant activity. Examples include putative effects on blood pressure, cell proliferation, monocyte function, vascular responsiveness and blood coagulation [48–52,60–64].

3. Where are we now?

At what stage are we now in understanding the impact of diet on oxidative damage in cardiovascular disease? Several studies have addressed the effects of diet (as opposed to antioxidant supplements) on lipid peroxidation in the human body. For example, diets rich in fruits and vegetables were reported to diminish ethane exhalation [65,66], urinary excretion of the isoprostane 8-epi-PGF_2α (but not of malondialdehyde) [39] and to decrease the susceptibility of low-density lipoproteins (LDL) to ex vivo peroxidation [66]. Patients participating in a programme of exercise plus a low-fat diet also had LDL with decreased susceptibility to peroxidation [67]. But were the indices of lipid peroxidation used in these studies valid? What is the best way to measure lipid peroxidation in human intervention studies?

4. Assaying lipid peroxidation

The criteria that should be met by the ‘ideal’ assay of lipid peroxidation are summarised in Table 1. No existing assay meets all these criteria, but some are better than others. First, lipid peroxidation should not be measured in human tissues or body fluids by simple diene conjugation methods or determinations of thiobarbituric acid-reactive material (TBARS), except where they have been previously calibrated against more sophisticated assays. The simple TBA test and diene conjugation methods are flawed (reviewed in [9,68]).

The peroxidation that contributes to atherosclerosis is thought to occur within blood vessel walls and not (or at least not to a large extent) in LDL circulating in the blood [15,16]. Just as LDL can enter vessel walls, minimally modified LDL (i.e. LDL that has undergone some oxidation, but insufficient for recognition by scavenger receptors) may escape back into the circulation. Therefore, one potentially useful biomarker perhaps indicative of peroxidation in blood vessels may be the susceptibility of circulating LDL to peroxidation. Indeed, this assay is widely used to show that antioxidants that inhibit lipid peroxidation in vitro might exert effects in vivo [69,70]. The dietary manipulation [66,67] or antioxidant supplement is administered to the subject and LDL is subsequently isolated and subjected to prooxidant challenge. This technique has been used by the group of Esterbauer and others [69,70] to investigate the optimal intake of vitamin E to make LDL most resistant to peroxidation. That intake turns out to be higher (>150 IU of RRR-α-tocopherol)
than can be achieved by relying on foods as a source of vitamin E. This is consistent with the epidemiological data; although there is considerable disagreement, the results of intervention trials suggest that the intakes of vitamin E that exert cardiovascular benefit in some studies are higher than can be obtained from diet alone [22,55,71]. The ex vivo LDL peroxidation method has also been used to examine the effects of consumption of wines and teas on LDL peroxidisability in humans: several papers suggest that tea has no effect [71±74] but reports for red wine are conflicting [75–79], although on balance suggestive of benefit.

An important question in studies of ex vivo LDL peroxidation is how to initiate the peroxidation. This is usually achieved by adding Cu$^{2+}$ ions. There is evidence that copper plays a role in LDL oxidation in vivo [80,81], but peroxynitrite, reactive chlorine species, haem proteins, hydroxyl radicals, and lipoxygenases could also be involved [81±86] and the predominant oxidizing species may differ from lesion to lesion or even in different regions of the same lesion. It is sensible to check several different methods (including incubating the LDL with endothelial and other cell types found in the vessel wall) of oxidising LDL in ex vivo studies before forming conclusions about the efficacy of administered antioxidants. For example, an antioxidant that inhibits Cu$^{2+}$-dependent LDL oxidation ex vivo by chelating Cu$^{2+}$ may not be effective in lesions in which the predominant LDL oxidation is achieved by other mechanisms. Another problem is that certain amphipathic antioxidants may protect LDL in the plasma environment but wash out during the prolonged procedures often used to purify LDL, so that they could exert protection in vivo but appear to exert no effect in ex vivo studies. This could conceivably happen with many plant phenolics, which have some degree of solubility in aqueous media.

A related approach is to measure the levels of circulating antibodies against LDL, which is presumably related to the extent of LDL oxidation in vivo [87,88]. It should be noted, however, that the precise relationship of LDL oxidation in the vessel wall to LDL oxidation in plasma remains to be clearly elucidated.

### 4.1. Direct assays of lipid peroxidation

In principle, a simple approach would be to measure products of lipid peroxidation in plasma or urine. Multiple methods have, for example, been used to measure lipid peroxide levels in human plasma. Simple colorimetric assays give values for plasma peroxides in the μM range (Table 2). Similar values are often given by TBA tests linked to HPLC to remove interfering chromogens [9,68]. By contrast, methods based on direct HPLC analysis of plasma peroxides produce lower values, usually <40 nM for the total of phospholipid and cholesterol ester hydroperoxides assayed by HPLC with coulometric or luminescence detection [89,90]. Is it that the simpler methods (Table 2) are non-specific, measuring other oxidizing species (e.g. protein peroxides [82]), or is it that the more complex methods degrade peroxides or otherwise fail to measure some of them during analysis [91]?

The GC–MS-based analysis of specific peroxides after reduction to alcohols is a chemically robust technology (Table 1) and gives information about the peroxidation of individual fatty acid residues [92,93]. For example concentrations of C18 hydroxyacid in plasma of healthy men and women were approximately 1 μM, whereas C20 hydroxyacid concentrations were 100–150 nM [92,93]. However, these methods will measure hydroxyacids (lipid alcohols) originally present in the biological material, in addition to those generated from the peroxides present by the reduction step prior to analysis. Other chemically
Table 2
Levels of lipid peroxides in human plasma or serum from healthy subjects: simple colorimetric assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>Typical level</th>
<th>Comment/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodine liberation</td>
<td>2.1±4.6 μM</td>
<td>Widely used in the food industry; Anal Biochem 1989;176:300; Atherosclerosis 1996;121:193. Can detect any species capable of oxidizing I⁻ to I₂.</td>
</tr>
<tr>
<td>Ferrous Oxidation Xylenol Orange (FOX)</td>
<td>3.0±4.0 μM (higher in some papers)</td>
<td>Levels elevated in diabetes. Can detect any species (including H₂O₂) that can oxidize Fe²⁺ to Fe³⁺; Anal Biochem 1994;220:403; Meth Enzymol 1999;300:58</td>
</tr>
<tr>
<td>Glutathione peroxidase-based assays</td>
<td>-1.0 μM</td>
<td>Chem Res Tox 1989;2:295</td>
</tr>
<tr>
<td>Activation of cyclooxygenase</td>
<td>-0.5 μM</td>
<td>Relates the presence of peroxides to one of their potential biological actions, stimulation of eicosanoid synthesis; Anal Biochem 1985;145:192 and 1991;193:55</td>
</tr>
<tr>
<td>Methylene blue method</td>
<td>8.6±5.8 μM</td>
<td>Atherosclerosis 1996;121:193; Exp Gerontol 1987;22:103</td>
</tr>
</tbody>
</table>

Robust methods include analysis of end-products of peroxide breakdown, such as malondialdehyde (MDA) and 4-hydroxyynonenal (HNE) by mass spectrometry [94,95]. Levels of 25–140 nM MDA have been reported in human plasma [94,96], whereas levels of HNE vary over a wide range and have sometimes been claimed to approach 1 μM (reviewed in [9,95]). These aldehydes result from breakdown of peroxides, and thus their levels will be influenced by rates of peroxide breakdown (which are slow at 37°C unless transition metal ions are present [9]), as well as by rates of metabolism of both peroxides and the aldehydes. There is evidence that peroxides and aldehydes in food can be absorbed through the gut to a limited extent and can potentially confound measures of MDA (especially in urine), TBARS, and perhaps of plasma peroxides [97–101].

The discrepancy between the different levels of lipid peroxidation products measured by different assays, and the question of confounding by diet, need resolution before any of the above methods can be recommended for studies of the effect of diet on lipid peroxidation rates in vivo. One approach to determination of ‘whole-body’ lipid peroxidation has been measurement of exhaled hydrocarbons, especially ethane [102–104]. Hydrocarbon gases are, however, minor end-products of peroxidation and their formation, like that of aldehydes, depends on the decomposition of peroxides, e.g. by transition metal ions [9]. Nevertheless, this assay has given some interesting results (e.g. Ref. [65]) and merits further exploration for use in intervention studies.

4.2. Isoprostanes

There is a growing belief that the most valuable of the currently available biomarkers of lipid peroxidation in the human body is the isoprostanes, which are specific products arising from the peroxidation of unsaturated fatty acid residues in lipids [19,20,101,105–120]. Some isoprostanes exist free in human plasma, but most are esterified to lipids. Isoprostanes can be accurately and sensitively measured by mass spectrometric techniques, so that steady-state levels in human body fluids can easily be detected [105,106,119,120]. Isoprostanes appear to turn over rapidly, being both metabolised and excreted [105,106,116]. Detection of them and their metabolites in urine may therefore be a useful assay of ‘whole-body’ lipid peroxidation [116,120]. As a ‘biomarker’ of lipid peroxidation, isoprostane analysis seems to fulfil many of the criteria listed in Table 2. One exception is that the isoprostanes usually measured (often 8-epi-PGF₂α) are only minor products of the peroxidation process. Several methodological questions relating to isoprostane analysis remain to be resolved (reviewed in [105]). The question of confounding by oxidized lipids in the diet has been examined experimentally, and there is no evidence as yet that measurements of plasma isoprostanes in humans are so confounded [101,121,122].

Most analyses of isoprostanes to date have focused on measurement of some of the F₂-isoprostanes, which arise from the peroxidation of arachidonic acid residues [106]. Levels of certain F₂-isoprostanes in human body fluids have been shown to be elevated in conditions that predispose to accelerated development of cardiovascular disease: diabetes [114,115], hypercholesterolaemia [107,109] hyperhomocysteinaemia [123] and cigarette smoking [108,110,112]. F₂-isoprostane levels in humans are responsive to dietary antioxidants (Table 3), although most studies have been on ‘unhealthy’ subjects with
Table 3

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Intervention</th>
<th>Change</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy volunteers</td>
<td>4 g/day vitamin C, 3200 IU/day vitamin E, 300 mg/day β-carotene, all for 2 weeks</td>
<td>37% fall in esterified F₃-Ips.</td>
<td>Drug Metab Rev 1999;31:117</td>
</tr>
<tr>
<td></td>
<td>400 IU α-tocopherol for 2 weeks</td>
<td>25% fall in unesterified F₃-Ips.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>800 IU α-tocopherol for 2 weeks</td>
<td>37% fall in unesterified F₃-Ips.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>400 IU per day α-tocopherol for 8 weeks, 600 mg per day of lipoic acid for 8 weeks</td>
<td>Both produced significant falls in urinary F₃-isoprostanes as measured by commercial immunoassay kit The combination was no better than each antioxidant individually</td>
<td>Free Rad Biol Med 1999;27:1114</td>
</tr>
<tr>
<td>Diabetic patients</td>
<td>600 mg/day vitamin E for 14 days</td>
<td>37% decrease in urinary levels</td>
<td>Circulation 1999;99:224</td>
</tr>
<tr>
<td>Smokers</td>
<td>100 or 800 U/day Vitamin E, 2 g/day vitamin C, both for 5 days</td>
<td>29% fall in urinary levels with C; 22% fall with the combination; no effect of E alone.</td>
<td>Circulation 1996;94:19</td>
</tr>
<tr>
<td>Hypercholesterolaemic patients</td>
<td>100 mg/day vitamin E for 2 weeks</td>
<td>34–36% fall in urinary levels.</td>
<td>Arterio Thromb Vasc Biol 1997;17:3230</td>
</tr>
<tr>
<td></td>
<td>600 mg/day vitamin E for 2 weeks</td>
<td>47–58% fall in urinary levels</td>
<td></td>
</tr>
<tr>
<td>Patients with anti-phospholipid antibodies</td>
<td>900 IU/day vitamin E, 2 g/day vitamin C, both for 4 weeks</td>
<td>Significant decrease in urinary levels</td>
<td>Blood 1999;93:3401</td>
</tr>
<tr>
<td>Patients with liver cirrhosis</td>
<td>300 mg vitamin E twice daily for 30 days</td>
<td>51% fall in urinary levels</td>
<td>Blood 1999;93:2945 (see also J Invest Med 1998;46:51)</td>
</tr>
<tr>
<td>Patients with chronic alcoholic liver disease</td>
<td>2.5 g vitamin C for 10 days</td>
<td>Approximately 50% fall in urinary levels</td>
<td>J Clin Invest 1999;104:805</td>
</tr>
</tbody>
</table>

5. Conclusion

Robust biomarkers of lipid peroxidation (of which at the moment the best available seem to be the isoprostanes) should be used to establish the effects of diet on lipid peroxidation in vivo, and in particular what contribution is made to any effect of diet by the antioxidants present in that diet (ascorbate, vitamin E, carotenoids, flavonoids etc.). Any cardiovascular disease intervention trial that does take place should be accompanied by measurements of lipid peroxidation. This will help to assess whether any beneficial effects observed on clinical end-points are related to inhibition of lipid peroxidation, and/or to other physiological effects [48–52,60–64] of antioxidants. Measurements of lipid peroxidation should be carried out:

1. Prior to the intervention study, to show (in short-term studies) that the intervention does actually decrease lipid peroxidation, preferably in a dose-dependent manner, in the subjects to be examined.
2. At intervals during the study, to relate changes in the levels of lipid peroxidation to clinical end-points.

One further point may be important. The ‘steady-state’ levels of oxidative DNA damage and lipid peroxidation vary widely between healthy human subjects. Are those with high levels of lipid peroxidation more at risk of...
developing cardiovascular disease later in life, and would antioxidants help to prevent this [31,132]?
Could it be that there is only a subset of the ‘healthy’ population who will benefit from antioxidant intervention [132]?
This subset might not be detected in a large intervention study because the subjects are ‘swamped out’ by subjects with low levels of ‘background’ peroxidation who respond minimally if at all to antioxidants [31,132,133]?
The combination of excellent chemistry (robust and validated biomarkers of oxidative damage) with excellent epidemiology will be a powerful tool to answer these questions [31].

References


