Free Radicals and Cardiovascular Disease

Barry Halliwell


Free radical reactions are involved in many human diseases. In some, they make a significant contribution to the disease pathology whereas in other diseases they may be of little or no significance. There is now good evidence that lipid peroxidation is involved in foam cell generation in atherosclerotic plaques and that dietary vitamin E may have an anti-atherosclerotic action. The relation of this to dietary intake of polyunsaturated fatty acids and antioxidants is discussed. The role of free radical reactions in reoxygenation injury after ischaemia is also discussed, with particular reference to the heart. The combination of electron spin resonance techniques and the use of antioxidants has shown that generation of free radicals during ischaemia-reoxygenation can damage the myocardium causing delayed recovery of contractile function ( "stunning") within 60 seconds of reoxygenation. The roles of xanthine oxidase and the endothelial derived relaxing factor in radical generation during reoxygenation injury are discussed.

In a previous paper in this Journal, I discussed the relationship of free radical reactions to human disease. There are certain diseases which are probably caused by increased free radical reactions, such as the tissue destruction produced by ionizing radiation, which is largely mediated by hydroxyl radical. Some or all of the symptoms of Keshan disease, chronic selenium deficiency, may be mediated by lack of active glutathione peroxidase. Severe vitamin E deficiency, as a result of disorders of intestinal fat absorption, produces neurodegeneration that may be mediated by increased rates of free radical reactions to the brain.

Hence there are some conditions in which free radicals are responsible for the origin of the disease.

However, in most diseases in which a role for free radicals has been suggested, they are not responsible for the origin of the disease, but contribute to the disease pathology at a later stage. Thus in rheumatoid arthritis and the adult respiratory distress syndrome the fundamental problem is excessive infiltration and activation of neutrophils, leading to an oxidative stress. Similarly, iron-dependent free radical reactions that take place after ischaemic or traumatic injury to the brain are secondary to the initial injury, but they may make a significant contribution to worsening that injury.1,2

If, in most human diseases, free radical reactions are secondary processes, then one must ask the obvious question as to whether they make a significant contribution to the disease pathology or whether they are just epiphenomena. The answer probably differs in different diseases.1,2 For example, the muscles of patients with muscular dystrophy contain increased end-products of free radical
reactions, but there is no good clinical evidence that using free radical scavengers to inhibit these reactions has any benefit. So in muscular dystrophy the tissue wasting produces increased free radical reactions which are probably insignificant in worsening the damage. One condition in which a role for free radical reactions seems to be likely is iron overload, which includes not only haemochromatosis and thalassaemia but also alcohol-related iron overload and malnutrition. Good evidence has come from research in Jamaica that some of the side-effects of protein-calorie malnutrition are mediated not only by the lack of calories and essential amino acids but also by iron overload and lack of essential antioxidants, such as glutathione (due to the low intake of sulphur-containing amino acids in the diet). Other diseases in which free radicals are important include rheumatoid arthritis, autoimmune disease and post traumatic degeneration of the brain and spinal cord.

Oxidative Damage to Tissues: Atherosclerosis

Oxidative stress can produce damage to many different cellular systems. Thus exposure of cells to such stress can produce DNA fragmentation, rises in the levels of 'free' Ca\(^{2+}\) ions in the cytosol, leading to activation of Ca\(^{2+}\)-dependent proteases that can cleave the cytoskeleton, and lipid peroxidation. The sort of damage that is done by oxidative stress in diseases to which oxidative stress makes a significant contribution can vary. In the case of atherosclerosis, there is now good evidence that lipid peroxidation occurs within atherosclerotic plaques and contributes to plaque development. This evidence comes from three lines of work. First, if you dissect out fresh human plaques and assay for lipid peroxides, they are present. Second, antibodies raised against peroxidized low density lipoproteins (LDL) bind to human atherosclerotic plaques, suggesting that peroxidized LDL is present. These antibodies detect products formed by reaction of the aldehydes generated when lipid hydroperoxides break down (such as malondialdehyde and 4-hydroxynonenal), with proteins. Indeed, one antibody that has been used detects the product formed when 4-hydroxynonenal reacts with the lysine residues of apoprotein B in the LDL. Third, advanced atherosclerotic lesions often contain the fluorescent pigment ceroid, which is an end-product of lipid peroxidation.

One of the characteristic features of human atherosclerotic lesions is the presence of large numbers of lipid-laden foam cells, many of which originate from macrophages. It seems very likely that a major mechanism leading to foam cell generation is the peroxidation of LDL. Macrophages possess receptors for natural LDL, which is taken up at a slow rate, but peroxidized LDL is taken up much faster by a different set of receptors (the scavenger receptors). Uptake of large amounts of peroxidized LDL by macrophages can lead to foam cell generation and development of the atherosclerotic plaque. How exactly peroxidation is initiated in the atherosclerotic plaque is uncertain. Lipoygenase enzymes may be involved. Alternatively, cell necrosis in the lesion may liberate iron ions which react with \(\text{H}_2\text{O}_2\) and \(\text{O}_2^-\) from macrophages to generate species such as hydroxyl radical, which can initiate peroxidation.

An area that is only just beginning to be explored is what happens to cholesterol when lipid peroxidation takes place. When peroxidation occurs in a membrane some of the reactive peroxyl radicals can react with the cholesterol present and oxidize it to a wide range of products, many of which have been shown to do considerable damage to vascular endothelium and to be highly toxic. The exact relation between products of cholesterol oxidation and the development of atherosclerotic lesions is not yet clear, but deserves more investigation since high plasma cholesterol levels are an established risk factor for atherosclerosis.

What factors govern the rate of LDL peroxidation in an atherosclerotic lesion? One is the content of polyunsaturated fatty acid side chains in the LDL lipids, since it is the polyunsaturated fatty acid side chains that peroxidize fastest. (Realization that polyunsaturated fatty acids are particularly prone to lipid peroxidation and that this process contributes to the growth of the atherosclerotic lesion has recently, at least in England, led to a re-evaluation of the suggestion that we should increase the amount of polyunsaturated fats in our diet. I think everyone is agreed we should eat less total fat, but not necessarily increase the amount of polyunsaturates). Another important factor is the content of \(\alpha\)-tocopherol, a chain-breaking antioxidant. A third factor is the content of carotenoids in LDL. In humans, carotenoids such as \(\beta\)-carotene and lycopene are absorbed through the gut and are transported in all the major classes of lipoproteins, including LDL.

How does \(\alpha\)-tocopherol protect against lipid peroxidation? As discussed previously, lipid peroxidation is a free radical chain reaction which is propagated by peroxyl radicals (lipid-O\(_2^-\)) abstracting hydrogen from adjacent fatty acid side chains. Peroxidation can be inhibited by adding any one of a class of molecules called chain-breaking antioxidants. Many of these molecules are phenols, containing an aromatic ring to which an \(-\text{OH}\) group is attached. They are usually hydrophobic molecules which can enter the non-polar interior of biological membranes. The phenolic \(-\text{OH}\) group is very good
at donating hydrogen. Hence peroxyl radicals formed during lipid peroxidation react with the chain breaking antioxidant (A-H) instead of reacting with an adjacent fatty acid side chain.

Lipid-$O_2^-$ + A-H → Lipid-$O_2H + A^-$

Chain-breaking antioxidants acting by this mechanism include $\alpha$-tocopherol, the most important chain-breaking antioxidant present in the human body, as well as several molecules that have been used as food preservatives, including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate, and nordihydroguaiaretic acid (NDGA). All of these, as well as $\alpha$-tocopherol itself, have been used in the food industry to protect food lipids against peroxidation.

There are many questions about the safety of synthetic antioxidants such as BHA and BHT. In a previous paper,$^1$ I mentioned a general principle of free radical reactions: they proceed as chain reactions, or 'radicals beget radicals'. The equation above illustrates this principle again. When the chain-breaking antioxidant combines with the peroxyl radical the antioxidant itself generates a radical (A'). However, studies of the toxicity of synthetic antioxidants have rarely taken this into account. There have been numerous toxicological studies in which animals are fed grotesque doses
of these compounds, but very few studies designed to answer the question 'does the radical that arises during the action of the antioxidant have any toxic properties?' Thus, in evaluating a synthetic chain-breaking antioxidant for use as a drug or food additive, one must ask if the antioxidant-derived radical might itself be able to do some damage, or at least find out what happens to it in vivo. The major physiological chain-breaking antioxidant α-tocopherol reacts with peroxyl radicals to make an α-tocopherol radical. There is good evidence that this radical can be reconverted to α-tocopherol at the surface of biological membranes by reacting with ascorbic acid (vitamin C). Thus ascorbic acid and α-tocopherol co-operate in helping to protect membranes against lipid peroxidation.

Figure 1 shows an experiment in which LDL isolated from human blood are placed under pro-oxidant conditions. The rate of peroxidation is measured as an absorbance increase at 234 nm, which essentially follows changes in the double bond structure of the fatty acids as they undergo peroxidation. The fate of the other constituents of the LDL was also followed. The pro-oxidant stimulus (a high concentration of copper ions) was applied at time zero, but there was a considerable lag period before significant peroxidation of the LDL was observed. During this lag period, the first change was that the tocopherols in the LDL disappeared. So tocopherols are the first line of defense: the α-tocopherol in the LDL is protecting them against peroxidation. Loss of tocopherols is followed by loss of β-carotene, lycopene, and phytoflavone. Not until all of these have disappeared does the peroxidation of LDL accelerate. So tocopherols seem to be the first line of defense and carotenoids also seem to contribute to protecting LDL against peroxidation.

If volunteers are given extra α-tocopherol for a few weeks before doing this experiment, more α-tocopherol enters the LDL and the lag period is extended quite considerably. Similarly, if volunteers are given the drug probucol, LDL subsequently isolated from them is also more resistant to peroxidation. Probucol was introduced as an agent that lowers plasma cholesterol concentrations, but it is also a powerful chain-breaking antioxidant, an activity that may account for part of its antiatherogenic action in experimental animals.

Figure 2 illustrates, again using isolated LDL, the ability of ascorbic acid to recycle α-tocopherols and protect LDL against peroxidation. Vitamin C added to the medium in which the LDL are suspended greatly increased the lag period. Hence the resistance of LDL to peroxidation will depend on one's dietary status: how much α-tocopherol and carotenoids are in LDL and how much ascorbic acid is in plasma.

Smoking predisposes to atherosclerosis, and, of course, there are several mechanisms that can account for this. Cigarette smoke is loaded with free radicals of many different kinds and it also activates phagocytes within the lung. Hence people who smoke are under an oxidative stress. Thus the alveolar lining fluid of cigarette smokers has, on average, less α-tocopherol than the alveolar fluid of non-smokers. Again, plasma levels of vitamin C and carotenoids tend to be lower in smokers than in non-smokers. In addition, cigarette smoke can damage the protein caeruloplasmin, a major antioxidant in plasma. Thus one potential mechanism by which cigarette smoking can predispose to atherosclerosis is by depleting levels of endogenous antioxidants such as α-tocopherol and vitamin C, so that peroxidation of LDL is promoted. It is often said that smokers need a higher intake of vitamin C, and possibly of α-tocopherol, than non-smokers.

Ischaemia-Reperfusion

The consequences of advanced atherosclerosis can be fatal: they include stroke and myocardial infarction. Formation of a thrombus at the site of an atherosclerotic lesion blocks an essential artery, the tissue is deprived of oxygen and the ischaemic tissue will eventually die. A very interesting point that is often not appreciated is that different body tissues differ enormously in their ability to survive 

O2 deprivation. The brain, after only short periods of ischaemia, undergoes irreversible injury, whereas skeletal muscle tolerates hours of ischaemia (limbs can be re-attached several hours after amputation, and a bloodless field is widely used in orthopaedic surgery). Human heart will probably tolerate something up to about 1 hour of ischaemia before injury becomes irreversible, irreversible injury meaning that if you restore the blood supply the tissue will never recover.

If, before a tissue has entered the irreversible injury phase, the blood supply is restored, tissue function will gradually recover. It is well known that if thrombus formation has taken place and a tissue has been deprived of oxygen, one must reperfuse the tissue as quickly as possible: hence the introduction of thrombolytic agents in the treatment of recent myocardial infarction.

Unfortunately reperfusion itself can sometimes create problems. Considerable evidence from studies with isolated hearts and experimental animals shows that reperfusion itself adds an additional insult to the tissue at the instant of reperfusion. Figure 3 illustrates one form of the so-called reperfusion injury. In an anaesthetized open chest dog, a branch of the coronary artery is occluded for 15 minutes, which deprives a portion of the myocardium of its blood supply. Fifteen
minutes is not long enough to injure the tissue irreversibly, so that tissue function will eventually recover when the blood supply is restored. However, the contractile function of the tissue does not return immediately the clamp is removed; it takes hours to return. This is the phenomenon known as myocardial stunning. Studies using the technique of electron spin resonance, which detects free radicals because it measures the presence of unpaired electrons, show that immediately upon reoxyg enation of the tissue (removing the clamp after 15 min) there is an intense generation of free radicals, which peaks at about 4 min after the blood supply is restored but continues at a significant rate for at least an hour. If certain antioxidants are added to the system these free radicals are no longer seen. The antioxidants effective in this system include the thiol compound mercaptopropionylglycine and the chelating agent desferrioxamine, which binds iron ions in forms incapable of stimulating free radical reactions. These compounds either scavenge the radicals or somehow prevent their formation.

Figure 3 shows that when the vessel is clamped, contractile function ceases and when the tissue is reperfused, contractile function comes back only very slowly. If mercaptopropionylglycine is injected into the reperfusing blood the contractile function comes back much faster, within a few minutes (Fig. 3). Only about half of the function returns, suggesting that the free radical generation accounts for only about half of the myocardial stunning phenomenon.

The protective effect of the mercaptopropionylglycine is the same whether it is present all the way through the experiment (i.e. during the occlusion and the reperfusion) or when it is added just at the time of reperfusion. However, if the mercaptopropionylglycine is injected 1 min after reperfusion has commenced, it has absolutely no protective effect even though it still interferes with free radical detection, as shown by the electron spin resonance signals. These data tell us that myocardial free radical production in this reasonably physiological system (the live animal is being perfused with its own blood and the model is the approximate equivalent of a patient undergoing open-heart surgery) occurs very quickly upon reperfusion, but that protection against reperfusion injury can only be achieved by removing these radicals extremely
quickly, within the first minute of the reperfusion. Similar results were obtained when mercaptopropionylglycine was replaced by desferrioxamine as the antioxidant.

The actual mechanism of radical generation during reoxygenation injury is unknown. The first proposal made was by McCord in the USA. When a cell is rendered ischaemic, ATP is degraded to produce hypoxanthine. Ischaemia also causes conversion of the enzyme xanthine dehydrogenase into xanthine oxidase, which generates $O_2^-$ and $H_2O_2$ when it acts upon its substrates. Hence ischaemia 'primes' the tissue for free radical generation because it causes both xanthine oxidase and its substrate hypoxanthine to accumulate in the tissue. When oxygen is restored the hypoxanthine is oxidized, producing $O_2^-$ and $H_2O_2$. This hypoxanthine/xanthine oxidase theory of reoxygenation injury seems to be a reasonable explanation for reperfusion injury in gut. However, there are many problems in applying this particular model to the heart, the organ in which we are particularly interested. Firstly, there may be no xanthine dehydrogenase or oxidase in the human heart or in the hearts of rabbit and pig, yet reperfusion injury and protection by free radical scavengers can be demonstrated in all of them. Debate continues in the literature as to whether the apparent lack of xanthine oxidase means that this enzyme is absent or is present in only one cell type, such as the endothelium.

However, our results (Fig. 3) suggest that if the free radical formation occurs instantly upon reoxygenation and has to be blocked within the first 60 seconds for protection to occur, one must be looking at something that is happening at the endothelial or myocyte cell surfaces. We cannot be looking at something that is happening deep within the tissue. I have mentioned previously that vascular endothelium produces small amounts of $O_2^-$, which can interact with nitric oxide endothelium-derived relaxing factor (EDRF), possibly as part of a vasoregulatory mechanism. Perhaps during ischaemia this physiological $O_2^-$ production mechanism is disrupted so that it greatly overproduces free radicals upon reperfusion. The initial product of reaction of $O_2^-$ with NO, peroxynitrite, may also cause cell damage. Another effect of ischaemia may be to make more iron available somehow, so that the $O_2^-$ production becomes damaging because iron ions are available to stimulate formation of reactive free radicals such as OH, upon reperfusion.

Conclusion

The final point I would like to make is to point out the complexity of tissue injury mechanisms in human disease. In the particular model of ischaemia/reperfusion injury that we use, we find a substantial protection by some antioxidants. Other models can give different degrees of protection. Just by slight changes in experimental conditions, one can change from a mechanism of tissue injury that is largely free radical-dependent, to something that is largely calcium ion-dependent. Human myocardial infarction patients are all different. They have had different periods of ischaemia. Some of them may have been treated with thrombolytic agents, some not, and so on. So to protect against ischaemia/reperfusion after thrombolyis I personally would not put all my bets on calcium blockers or free radical scavengers. What one could do is to protect against all potential mechanisms of cell injury simultaneously. Polypharmacy is not encouraged, but in fact quite often a single drug may act by multiple mechanisms. For example captopril, an angiotensin converting enzyme inhibitor, also has some free radical scavenging properties. So many of the drugs in clinical use may well be doing several different things. Drugs with multiple mechanisms of action, including antioxidant properties, may be the way forward in minimizing tissue injury in human disease.

References


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