established, the mechanism by which β-carotene exerts a protective function against cancer remains unknown. However, there are several lines of evidence which suggest that the generation of reactive oxygen species may play an important role in the development of cancer [14]. The present work emphasizes that attention should be extended from β-carotene to lycopene and other carotenoids. Lycopene has a plasma concentration slightly higher than β-carotene and both these carotenoids were found in low-density lipoproteins [15].

The relative physical quenching abilities of the tocopherol homologues decreased in the following order: α, β, γ, and δ-tocopherol. With the tocopherols, the ability of O₂⁻ quenching depends on a free hydroxyl group in position 6 of the chromate ring. Chemical reactivity of the tocopherol homologues were low, accounting for 0.1 to 1.5% of the physical quenching.

Among the biological thiols, cysteine was the most effective quencher of O₂⁻ followed by lipofuscin (disulfide form of the dithiol lipofuscin), coenzyme A, glutathione, cysteamine and dihydrolipoate. Pharmacologically active thiols like N-acetylcysteine, mesna, WR-1065 and captopril significantly differed in their quenching abilities. The pD dependence of the chemical quenching indicated that O₂⁻ reacts with the thiolate anion. As compared to their overall quenching abilities, cysteine, WR-1065, cysteamine, mesna and dihydrolipoate did not result in significant chemical quenching abilities. Other thiols tested had a chemical quenching rate less than 1%, of their overall quenching ability.

Compared to carotenoids, other classes of compounds, e.g. bilirubin, tocopherols and thiols were less active in singlet oxygen quenching. But these may also be biologically important in O₂⁻ quenching because of their higher concentration and/or different subcellular location in biological targets, besides solubility characteristics.

Our studies were supported by the National Foundation for Cancer Research, Bethesda, U.S.A., Kernforschungsanlage Jülich GmbH, Bhabha Atomic Research Centre, Bombay and by Deutsche Forschungsgemeinschaft, Bonn.


Received 16 July 1990

Free radicals, myocytes and reperfusion injury

JEREMY J. O. TURNER,*
CATHERINE A. RICE-EVANS,* MICHAEL J. DAVIES† and EMMA S. NEWMAN
†Department of Biochemistry, Royal Free Hospital School of Medicine, London NW3 2PF and ‡Department of Chemistry, University of York, York YO1 5DD, U.K.

There are several clinical settings in which the myocardium is exposed to transient ischaemia including evolving myocardial infarction, myocardial stunning and coronary thrombosis. On reperfusion, the sudden re-introduction of normotensive molecular oxygen may be detrimental to the previously ischaemic myocardium leading to suboptimal myocardial salvage. The myocardial response to ischaemia is highly dependent on the extent and duration of the ischaemia and the severity of coronary flow reduction.

Evidence for free radical involvement in reperfusion injury

There is much direct and indirect evidence for the contribution of radicals species to myocardial damage. The direct evidence comes from the application of techniques such as e.p.r. spectroscopy [1–3] which has confirmed the involvement of free radicals in in vivo animal models of coronary occlusion as well as in many isolated heart studies. Indirect evidence arises from protection afforded by specific scavengers of oxygen radicals and inhibitors of putative radical-generating systems in reducing infarct size and post-ischaemic contractile dysfunction. Recently the studies of Bolli et al. [3] have investigated the time window during which free radicals are generated in an open-chest dog model in vivo. The thiol-containing antioxidant compound, N-mercaptopropionyl glycin, was administered as an intracoronary infusion to dogs undergoing 15 min coronary occlusion and the drug infusion started at various specific time-points before and after reperfusion. Assessment of recovery of contractile function in terms of wall thickening and of inhibition of free radical production by e.p.r. after intracoronary infusion of a spin trap indicates that most of the damage responsible for myocardial stunning develops in the initial seconds after reperfusion and can be prevented by antioxidant therapy started just below reflow.

In addition, earlier studies of others [4–8] had shown attenuation of the incidence of arrhythmias and other markers of reperfusion damage by anti-radical interventions in a range of animal models. Such compounds included superoxide dismutase and catalase, several hydroxyl radical scavengers and the iron chelator desferrioxamine. All of these studies suggest that attenuation of these events by incorporation of appropriate anti-radical interventions in combination with thrombolytic therapy, for example, may help overcome the cellular damage that occurs secondarily to the initial pathology in the clinical condition.

Thus, a detailed understanding of the processes leading to the radical-dependent pathology in reperfusion injury, as well as the nature and sources of the toxic species, are crucial for the design of effective intervention strategies.
Potential sources of free radicals in reperfusion injury

There are several potential sources of free radicals during myocardial post-ischaemic reperfusion injury which are inter-related in a complex fashion. These have been the subject of many recent reviews [9-11].

1. Oxygen-derived free radicals are produced by activated neutrophils which infiltrate the ischaemic and reperfused myocardium [12,13]. The neutrophil and other phagocytic cells possess the capacity to produce reactive species of oxygen through the respiratory burst when activated by components of the complement system, for example. The ischaemic myocardial tissue gives rise to a tissue protease that activates complement [14] and ischaemia results in migration of neutrophils into the primed myocardium. Other chemotactic agents including leukotriene B4 are also generated in response to myocardial tissue injury. Reperfusion of the previously ischaemic myocardium allows for rapid access of the inflammatory cells to the primed myocardial region at risk [15]. The oxidants produced by activated phagocytes consist of superoxide radical (O2•−), hydrogen peroxide (H2O2), hypochlorous acid (HOCI) and chloramines which normally function to provide a defence mechanism against microorganisms. The reactive oxygen species have deleterious effects on surrounding cells and tissues when they are released into the extracellular environment.

2. An additional potential source of oxygen-derived free radicals is the enzyme xanthine oxidase [9,16]. This enzyme is localized within the vascular endothelial cells but there is a controversy as to whether it is present in the myocytes of the human heart. Xanthine dehydrogenase is converted to xanthine oxidase on activation of a calcium-dependent protease by increased calcium levels made available from the changes in metabolism occurring during the ischaemic period. This observation is supported by the reduction in tissue injury on reperfusion in the presence of allopurinol [17].

3. Intracellularly, radicals may also arise during leakage from the mitochondrial electron transport chain as a consequence of the accumulation of reducing equivalents during the ischaemic period [18].

4. An aspect of particular interest to us relates to the haem proteins as potential sources of radicals in vivo. Interaction of haem proteins with hydrogen peroxide, derived from oxygen species generated during reperfusion, leads to the activation of haem proteins to ferryl states which have been characterized as free radical entities, under specific conditions; furthermore, at certain levels of hydrogen peroxide in excess of the haem proteins, with prolonged exposure, destabilization of the haem ring occurs and iron may be released. This mechanism may have implications for events within the myocyte as well as those that may occur extracellularly. It is now recognized that myoglobinemia, arising from the rupture of myocytes, is the earliest detectable marker of acute myocardial infarction [19], thus creating a potential scenario for the involvement of haem proteins in radical production. In addition, the myocyte contains glutathione peroxidase [20] but relatively very little catalase [21]; thus loss or impaired function of intracellular antioxidants arising during the ischaemic phase predisposes the myocardium to further injury upon reperfusion by reducing the ability of the cell to defend itself against the deleterious effects of elevated radical formation.

Hence, the attachment and activation of neutrophils at a site of injury on the endothelium after ischaemic insult, in addition to intracellular sources of reactive oxygen species and the delocalization of haem proteins may all participate in the tissue injury associated with the onset of reperfusion, which is additive to the myocardial damage and secondary to the ischaemic insult itself.

What is the precise nature of the radical species contributing towards tissue damage in reperfusion injury?

Many cellular constituents including enzymes, ion channels, structural proteins and membranal lipids are potential targets for radical-mediated cellular injury. Such damage may lead to the compromising of a range of cellular functions and, ultimately, may lead to cell death, thereby contributing to the rhythmic and contractile dysfunctions observed following myocardial ischaemia-reperfusion.

In this work we focus on the nature and origins of the radical species contributing towards reperfusion injury and, in particular, on the relevance of decompartmentalized iron proteins. Rupture of myocytes in the acutely infarcted myocardium is a well-documented event; thus, biochemical investigations of the potential for the involvement of cardiac myocytes, under oxidative stress, in radical generation and the nature of the resulting species were undertaken.

Interaction of isolated cardiac myocytes with hydrogen peroxide results in a stimulation of lipid peroxidation [21], as detected by the thiobarbituric acid assay in these cells. Formation of radical species capable of initiating this process, in response to oxidative stress imposed by hydrogen peroxide, is therefore indicated. Possible identities of such a species include the hydroxyl radical and ferryl haem protein radicals.

It has been suggested that haem proteins are potential candidates for hydroxyl radical formation in vivo [22]. Possible explanations for this include the ability of haemoglobin to release iron on exposure to excess concentrations of hydrogen peroxide or lipid hydroperoxides [23], and the release of iron from myoglobin on interaction with excess hydrogen peroxide, the generation of hydroxyl radical being measured by its ability to degrade deoxyribose [22].

In the presence of less excessive concentrations of hydrogen peroxide, however, myoglobin stimulates lipid peroxidation [24,25] and several workers have shown that the radical species generated is akin to that designated as ferryl myoglobin [26-28] in which the haem iron is one oxidizing equivalent above that of myoglobin and one oxidizing equivalent is accepted by the globin moiety.

\[\text{HX-Fe}^{IV} + \text{H}_2\text{O}_2 \rightarrow \text{X-}[\text{Fe}^{IV}=\text{O}] + \text{H}_2\text{O}\]

The tyrosine phenoxyl radical thereby formed appears to react subsequently with oxygen to give a tyrosine peroxyl radical. Both of these species are located on the surface of the protein [29] and are accessible for participation in radical reactions, such as membrane lipid [24,25] and lipoprotein [30] peroxidation, and to electron-donating antioxidants. Additional peroxidative damage may result, at higher levels of oxidant concentration and prolonged exposure, from hydrogen peroxide-induced release of iron from the haem protein and subsequent generation of hydroxyl radical. However, under such conditions, iron release from myoglobin may be modulated by electron-donating antioxidants [25], as well as membrane lipids [25] which suppress ferryl myoglobin formation, thereby becoming targets for ferryl radical-mediated peroxidative degradation.

E.p.r. investigations of cardiac myocytes exposed to hydrogen peroxide were undertaken in order to characterize the nature of the radical species which may be participating in the observed cellular damage. Utilizing the spin trap 5,5'-dimethyl-1-pyrroline N-oxide (DMPO), a signal with features in common with that previously assigned to the adduct of the tyrosine peroxyl radical is observed indicating that a similar process to those observed with activated metmyoglobin and oxymyoglobin is occurring (Fig. 1). A similar signal has not been detected with other haem proteins suggesting that activated cytochrome proteins from the mito-
destruction during post-ischaemic reperfusion injury, from chelators connection it has been reported that incorporation of iron oxides.

In the myocyte the red shift in oxymyoglobin is at 414 nm and that of ferryl myoglobin at 420 nm. In the myocyte the red shift in response to exposure to hydrogen peroxide, characteristic spectral transitions occur, both in the Soret region and at longer wavelengths. We have characterized the nature of the former transitions occurring in one of the major haem proteins present in the myocyte, myoglobin. While the Soret peak of metmyoglobin occurs at 410 nm, that of oxymyoglobin is at 414 nm and that of ferryl myoglobin at 420 nm. In the myocyte the red shift in the position of this peak in response to hydrogen peroxide is interpreted in terms of the formation of the activated ferryl myoglobin species, which concurs with our e.p.r. observations. Consistent with this, Walters et al. [31] detected the formation of ferryl myoglobin in isolated cardiac myocytes in vitro in response to oxidative stress imposed by hydroperoxides.

The role of desferrioxamine

As mentioned earlier, many studies have emphasized iron-mediated generation of hydroxyl radicals as potential contributory factors in the pathogenesis of myocardial tissue destruction during post-ischaemic reperfusion injury, from evidence provided by both in vitro and in vivo studies. In this connection it has been reported that incorporation of iron chelators [32–35], and desferrioxamine is of specific interest here, protects against such radical damage by the proposed mechanism of the chelation of available iron.

However, recent observations show that desferrioxamine has activities other than as an iron chelator [25, 36–40] and its ability to enter, on a physiologically relevant timescale [41], only those cells undergoing active pinocytosis (J. Lloyd, H. Cable & C. Rice-Evans, unpublished work) suggests that the interpretation of some of the results invoking hydroxyl radical involvement may not be so clear cut and alternative considerations could be significant. For example, recent work has proposed that the trihydroxamate moiety of desferrioxamine, the centre involved in the binding of the ion, can be involved in electron transfer through its ability to donate atoms or electrons to a variety of systems involving haem proteins activated by hydrogen peroxide such as ferryl myoglobin, activated horseradish peroxidase as well as to superoxide radical [25, 36–38]. In addition, the ability of desferrioxamine to intercept the propagation phase of peroxidizing membrane lipids has also been demonstrated [25, 39, 40] with the formation of the desferrioxamine nitroxide radical [40].

Conclusions

Thus, cardiac myocytes under oxidative stress, in vitro, have the ability to form radical species, detectable by e.p.r. and visible spectroscopy, which are capable of inducing peroxidative damage to cell membranes, specifically myocyte membranes and erythrocyte membranes. It is suggested that these species include ferryl myoglobin radicals.

It should also be noted that after prolonged exposure of the myocytes to oxidative stress over the course of 5 h significant haem protein destabilization was indicated. Under such conditions, it is conceivable that iron may subsequently become available, providing the potential for hydroxyl radical formation, though this may be a very late event when irreversible damage has already occurred in the myocytes. The nature of the radical species involved may therefore be influenced by the severity and the duration of the oxidative stress to which the myocytes are exposed. The recognition of the ability of desferrioxamine to function as an electron donor, quite independently of its iron chelation properties, and hence to exhibit antioxidant activities, including the suppression of ferryl myoglobin and the interception of the propagation phase of peroxidizing membrane lipids suggest that the ability of this agent to limit myocardial ischaemia/reperfusion injury in animal models may have other interpretations.

For financial support we thank the British Technology Group, the Wolfson Foundation and the Wellcome Trust. We acknowledge the S.E.R.C. for provision of e.p.r. facilities (University of York).

Endogenous antioxidants and lipoprotein oxidation

HERMANN ESTERBAUER, MARTINA DIEBER-ROTHENEDER, GEORG WAEG, HERBERT PUHL and FRANZ TATZBER

Institute of Biochemistry, University of Graz, A-8010 Graz, Austria

Human low-density lipoprotein (LDL) is not only rich in cholesterol, but also in polyunsaturated fatty acids (PUFAS) susceptible to lipid peroxidation (Table 1). Various lines of biochemical, clinical and epidemiological studies suggest that oxidatively modified LDL (oLDL) is atherogenic and that preventing LDL oxidation by antioxidants could diminish the risk of ischemic heart diseases (for review see [1-3]). LDL particles oxidized in vitro or in vivo, e.g., in diabetic or hypercholesterolemic subjects, are more susceptible to scavenger receptor binding than non-oxidized LDL, and their uptake in macrophages is enhanced. oLDL stimulates monocyte chemotaxis and enhances the expression of the scavenger receptor on monocytes which phagocytize oLDL. In vivo administration of oLDL to hypercholesterolemic rabbits increases the atherosclerotic lesion size in the aortic arch. Furthermore, oLDL stimulates monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) in monocytes/macrophages. This is consistent with our findings that the scavenger receptors on monocytes/macrophages are upregulated in atherosclerotic lesions and activated atherosclerotic plaques. The scavenger receptor may be a link between oLDL and the development of atherosclerosis.

The process of Cu²⁺-stimulated oxidation can be divided into two major phases: (1) the initiation phase, during which the endogenous antioxidants contained in LDL are depleted, and when the LDL is depleted from all its antioxidants, it is, as expressed by Brown & Goldstein, "left to the mercy of oxygen" [7]. That means that the lipid peroxidation process enters into the propagating phase; (2) the propagation phase is characterized by a rapid oxidation of the PUFAS to lipid-hydroperoxides. The increase of the lipid peroxides can be measured by iodometric assays or, more conveniently, by the continuous recording of the increase of the characteristic absorption at 234 nm (Fig. 1), which develops through the formation of hydrogen peroxide with the conjugated double bond system CH=CH-CH=CH(0OH)--; (3) the decomposition phase, in which the lipid peroxides decompose to a great variety of products. A number of reactive aldehydes were identified, such as malonaldehyde, 4-hydroxynonenal, 4-hydroxyhexenal, 4-hydroxyoctenal, propanal, butanal, n-propienal, n-butanal, n-valeraldehyde, 2-n-propylfuran, but-3-enal, isoprene, isoprene oxide, and hydroxynonenal. The formation of these aldehydes is consistent with the hydroperoxide structure of oLDL, and suggests that oLDL is a good model for the study of human atherosclerotic lesions.

Table 1. Basal values of cholesterol, PUFAS, antioxidants and lag phases determined for human LDL samples

<table>
<thead>
<tr>
<th>Total LDL (nmol/mg)</th>
<th>n</th>
<th>Mean ± S.D</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>6</td>
<td>800 ± 90</td>
<td>750-1000</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>14</td>
<td>424 ± 107</td>
<td>272-690</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>14</td>
<td>54 ± 30</td>
<td>19-100</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>67</td>
<td>2.59 ± 0.70</td>
<td>1.26-5.96</td>
</tr>
<tr>
<td>γ-Tocopherol</td>
<td>67</td>
<td>0.22 ± 0.08</td>
<td>0.09-0.54</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>64</td>
<td>0.12 ± 0.08</td>
<td>0.02-0.36</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>63</td>
<td>0.06 ± 0.04</td>
<td>0.01-0.06</td>
</tr>
<tr>
<td>Cryptoxanthine</td>
<td>37</td>
<td>0.05 ± 0.03</td>
<td>0.02-0.14</td>
</tr>
<tr>
<td>Zeaxanthine/Lutein</td>
<td>36</td>
<td>0.02 ± 0.01</td>
<td>0.01-0.06</td>
</tr>
<tr>
<td>Lag phase (min)</td>
<td>73</td>
<td>74.6 ± 21.8</td>
<td>44-151</td>
</tr>
</tbody>
</table>

Abbreviations used: LDL, low-density lipoprotein; oLDL, oxidatively modified LDL; PUFAS, polyunsaturated fatty acids; ROR, relative oxidation resistance.

Vol. 18