Cardioprotection against ischaemia/reperfusion by vitamins C and E plus n−3 fatty acids: molecular mechanisms and potential clinical applications

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Abstract
The role of oxidative stress in ischaemic heart disease has been thoroughly investigated in humans. Increased levels of ROS (reactive oxygen species) and RNS (reactive nitrogen species) have been demonstrated during ischaemia and post-ischaemic reperfusion in humans. Depending on their concentrations, these reactive species can act either as benevolent molecules that promote cell survival (at low-to-moderate concentrations) or can induce irreversible cellular damage and death (at high concentrations). Although high ROS levels can induce NF-κB (nuclear factor κB) activation, inflammation, apoptosis or necrosis, low-to-moderate levels can enhance the antioxidant response, via Nrf2 (nuclear factor-erythroid 2-related factor 2) activation. However, a clear definition of these concentration thresholds remains to be established. Although a number of experimental studies have demonstrated that oxidative stress plays a major role in heart ischaemia/reperfusion pathophysiology, controlled clinical trials have failed to prove the efficacy of antioxidants in acute or long-term treatments of ischaemic heart disease. Oral doses of vitamin C are not sufficient to promote ROS scavenging and only down-regulate their production via NADPH oxidase, a biological effect shared by vitamin E to abrogate oxidative stress. However, infusion of vitamin C at doses high enough to achieve plasma levels of 10 mmol/l should prevent superoxide production and the pathophysiological cascade of deleterious heart effects. In turn, n−3 PUFA (polyunsaturated fatty acid) exposure leads to enhanced activity of antioxidant enzymes. In the present review, we present evidence to support the molecular basis for a novel pharmacological strategy using these antioxidant vitamins plus n−3 PUFAs for cardioprotection in clinical settings, such as post-operative atrial fibrillation, percutaneous coronary intervention following acute myocardial infarction and other events that are associated with ischaemia/reperfusion.

Key words: antioxidant, apoptosis, autophagy, ischaemia/reperfusion, necrosis, oxidative stress.

INTRODUCTION
Coronary heart disease is the most common cause of death in the Western world, and it is likely that its incidence will increase in the near future [1]. Major advances in the treatment of acute coronary syndromes and MI (myocardial infarction), such as blood flow restoration using PCI (percutaneous coronary intervention) or intravenous thrombolytic drugs, have occurred over the past 20 years. Paradoxically, the process of ischaemia followed by reperfusion is often associated with the activation of an injurious cascade, thus leading to new myocardial challenges accounting for up to 50% of infarct size. Considerable evidence implicates ROS (reactive oxygen species) and RNS (reactive nitrogen species) species as causes of injury in clinical settings associated with myocardial oxidative stress [2]. The damage occurs during both ischaemia and post-ischaemic reperfusion in humans.
Moreover, available evidence suggests that these molecules can trigger defensive mechanisms essential for cell survival. It is accepted that the beneficial or deleterious effects of these reactive species are dependent on their low-to-moderate or high concentrations respectively, but a clear definition of these concentrations remains to be established. In experimental models, prior to reperfusion, ischaemic cultured cardiomyocytes produce significant levels of ROS from the residual O$_2$, which significantly contribute to cellular injury following reperfusion [3]. These events may have important consequences for cellular function and viability. In addition, levels of lipid peroxides and 8-isoprostaglandin F$_{2\alpha}$, the major biochemical markers of ROS generation, are elevated in the plasma and pericardial fluid of patients with heart failure and are positively correlated with the severity of this disease [4]. Furthermore, ischaemia also causes alterations in ROS defence mechanisms [5]. The present review focuses on the cellular and molecular mechanisms of oxidative-stress-induced myocardial damage during acute ischaemia and reperfusion and examines their viability as therapeutic targets for the antioxidant effects of vitamins C and E plus $n$ – $3$ PUFAs (polyunsaturated fatty acids).

**OXIDATIVE STRESS AND THE HEART**

**Sources of ROS**

ROS can be generated in a variety of ways in cardiac myocytes. The greatest ROS source is from electrons lost during the transfer between electron transport chain complexes in mitochondrial oxidative phosphorylation processes. Furthermore, ROS can be enzymatically generated from phagocytic NADPH oxidase, uncoupled eNOS (endothelial nitric oxide synthase), XO (xanthine oxidase), lipooxygenase/COX (cyclo-oxygenase), myeloperoxidase and the oxidation of catecholamines. NADPH oxidase activity also occurs in cardiomyocytes and heart endothelial cells. The phagocytic NADPH oxidase NOX2 (gp91) produces superoxide anions by reducing O$_2$ [6]; local non-phagocytic NADPH oxidases have important consequences for cellular function and viability. Furthermore, ischaemia also causes alterations in ROS defence mechanisms [5]. The present review focuses on the cellular and molecular mechanisms of oxidative-stress-induced myocardial damage during acute ischaemia and reperfusion and examines their viability as therapeutic targets for the antioxidant effects of vitamins C and E plus $n$ – $3$ PUFAs (polyunsaturated fatty acids).

**Antioxidant defences**

Enzymatic antioxidant defences include SOD (superoxide dismutase), GSH-Px (glutathione peroxidase) and CAT (catalase). Non-enzymatic antioxidants include a variety of biological molecules, such as ascorbic acid (vitamin C), $\alpha$-tocopherol (vitamin E), reduced glutathione (GSH), carotenoids, flavonoids, polyphenols and other exogenous antioxidants [13]. Oxidative stress develops when ROS production is sufficient to overwhelm the antioxidant defence system, which further increases the ROS and RNS steady-state concentrations and damages biomolecules. Examples of pathophysiological cardiac states associated with ischaemia and reperfusion are AMI (acute MI), cardiac surgery with extracorporeal circulation and heart transplantation. The vulnerability of myocardial tissue to oxidative challenges caused by reperfusion is dependent on antioxidant system activity. The first line of cellular defence against oxidative injury include the antioxidant enzymes CAT, SOD and GSH-Px. MnSOD (manganese SOD) overexpression protects the murine myocardium from post-ischaemic injury; however, neither GSH-Px nor Cu,ZnSOD (copper/zinc SOD) appear to be important determinants of the extent of myocardial injury in this *in vivo* model [8]. Consistent with this view, it has been reported that infarct size is markedly reduced in transgenic mice that overexpress SOD [14].

Exposure to low-to-moderate ROS levels should trigger a survival response and reinforce ROS scavengers of the antioxidant defence system to elicit a cardioprotective effect for myocardial reperfusion. In fact, the molecular mechanism responsible for this adaptive change involves enhanced antioxidant activity achieved by up-regulating several housekeeping genes partly under the control of Nrf2 (nuclear factor-erythroid 2-related factor 2). Nrf2 is normally sequestered in the cytosol by Keap1 [Kelch-like ECH (erythroid cell-derived protein with cap n’ collar homology)-associated protein 1]. Upon oxidative stimulation, Nrf2 oxidizes or covalently modifies Keap1 to release Nrf2 from target gene promoters [15], which increase the expression of antioxidant enzymes. It has been demonstrated that the constitutive levels/activities of a number of important antioxidants and phase 2 enzymes, such as CAT, GSH-Px, glutathione reductase, GST (glutathione transferase), NAPDH:quinoide oxidoreductase 1, and haem oxygenase-1, in primary cardiomyocytes are dependent on...
Nrf2 status. Nrf2 diminishes the susceptibility of cardiomyocytes to injury elicited by oxidants and electrophilic species [16], making the Nrf2 signalling pathway an important mechanism for myocardial cytoprotection.

It is of interest to note that ROS levels could be responsible for the activation of NF-κB and/or Nrf2 pathways. These responses are shown in Figure 1.

**INFLAMMATION IN ISCHAEMIA/REPERFUSION**

In addition to ROS release following heart ischaemia, the early reperfusion phase is characterized by enhanced cytokine and adhesion molecule expression. During the first hour of reperfusion, superoxide triggers neutrophil infiltration, which increases cardiac damage by further release of ROS, inflammatory mediators and proteases [17]. Myeloperoxidase, an inflammatory marker accounting for neutrophil accumulation, is also able of increasing ROS production via hypochlorous acid breakdown. Recently, it has been demonstrated that elevated serum myeloperoxidase activities are significantly associated with the prevalence of acute coronary syndrome in patients with coronary heart disease [18]; however, inhibition of this enzyme with ulinastatin [19] or phloroglucinol [20] protected the heart against ischaemia/reperfusion injury in the rat. Chemokines are known to induce leucocyte migration, growth and activation. Specifically, the chemokine IL (interleukin)-8 appears to have a fundamental role in regulating neutrophil localization in ischaemic myocardium. In mice, CXCL2 (CXC chemokine ligand 2), the homologue of human IL-8, is up-regulated in reperfused myocardium [21]. The chemokine response in ischaemic tissues may be induced by various factors, including ROS, cytokines [e.g. TNF (tumour necrosis factor)-α], complement and NF-κB activation [22]. Prospective epidemiological studies have shown that serum levels of CRP (C-reactive protein), a biomarker of inflammation, is a strong predictor of cardiovascular ischaemia/reperfusion injury cycle events, such as MI, post-operative AF (atrial fibrillation) and stroke [23]. Several studies have revealed an independent association of high plasma CRP levels with adverse prognosis in patients with AMI. Interestingly, pre-conditioning was found to inhibit post-ischaemic CRP increases in a rat model of AMI [24].
CELL DEATH IN ISCHAEMIA/REPERFUSION

Apoptosis
Apoptosis is a highly controlled cell death process that is autonomously committed by both healthy and sublethally injured cells in response to physiological or pathological stimuli, including ischaemia/reperfusion events. Necrotic cell death is a widely recognized property of ischaemic cell death and is clinically diagnosed by documenting myocyte release of cytosolic constituents, such as creatine kinase MB, troponins and other proteins. However, apoptosis has only been implicated in the pathogenesis of several acute and chronic conditions affecting the cardiovascular system in the last decade [25]. Whether myocyte apoptosis is initiated during ischaemia but dependent on reperfusion or if it is a feature of reperfusion injury requires further study. Reperfusion appears to accelerate apoptosis when compared with permanent occlusion [26]. The process includes extrinsic and intrinsic pathways.

Extrinsic pathway
The extrinsic pathway of apoptosis is specialized in transducing signals from soluble and cell-bound death ligands, such as FasL (Fas ligand) and TNF-α, which bind and activate their cognate cell-surface death receptors. The binding of Fas ligand triggers a conformational change in Fas that allows activation of its intracellular death domain and pro-caspases. Both soluble and membrane-bound Fas and FasL molecules exert a wide range of pro-inflammatory functions and are involved in early ventricular remodelling. It has been reported that cardiac overexpression of the FasL results in accentuated apoptosis in vitro, whereas lpr (lymphoproliferative) mice, which lack Fas, display less apoptosis and reduced infarct size in ischaemia/reperfusion studies [27].

Intrinsic pathway
The intrinsic pathway of apoptosis is responsible for transducing most apoptotic stimuli, including those due to inadequate nutrients/survival factors, hypoxia, oxidative stress, nutrient stress, DNA damage and cellular toxins. These stimuli converge at the mitochondria and mPTP (mitochondrial permeability transition pore) to trigger the release of cytochrome c, leading to the formation of the ‘apoptosome’ complex, which results in caspase 9 activation followed by caspase 3 activation [28]. Smac (second mitochondrial-derived activator of caspase)/DIABLO (direct inhibitor of apoptosis-binding protein with low pi) indirectly activates caspases by sequestering caspase-inhibitory proteins, whereas the mitochondrial release of endonuclease-G and apoptosis-inducing factor results in their translocation into the nucleus where they directly or indirectly facilitate DNA fragmentation [29]. Active caspases cleave vital substrates in the cell, such as actin, actinin, β-myosin heavy chain, myosin light chain, tropomyosin and cardiac troponins, leading to cellular demise [30]. Whether myocyte apoptosis is initiated during ischaemia but dependent on reperfusion or if it is a feature of reperfusion injury requires further study. Reperfusion appears to accelerate apoptosis when compared with permanent occlusion [26]. In contrast with the modest, chronically elevated levels of cell death during heart failure, MI is characterized by a large burst of cardiac myocyte death that is usually complete within 24 h.

Cross-talk between extrinsic and intrinsic pathways
The extrinsic pathway requires a close communication at the mitochondrial level. This connection is mediated by cleavage of the BH3-only proteins, which are a pro-apoptotic subgroup of the Bcl-2 family. Bcl-2-related proteins are currently the most relevant anti-apoptotic modulators. Bcl-2s not only function as anti-apoptotic proteins, but also as anti-autophagy proteins via their inhibitory interaction with Beclin 1. Therefore Bcl-2 may help maintain autophagy at levels that are compatible with cell survival, rather than cell death. In contrast, short peptides derived from the BH3 region possess intrinsic pro-death activity. Furthermore, certain peptides of this group exert their pro-death function by specifically binding Bcl-2 and opposing its anti-death function, as BH3 domains are prototype Bcl-2 inhibitors. In response to apoptotic stimuli, Bax undergoes activation and translocation to the mitochondria. A variety of Bax-binding proteins regulate the conformational change of Bax, although this process has not been well characterized [31,32]. In contrast with the general involvement of Bax, BH3-only proteins transduce cell signals in a stimulus-specific manner [33].

Necrosis
Necrotic cell death is a widely recognized property of ischaemic cell death and is clinically diagnosed by documenting myocyte release of cytosolic constituents, such as creatine kinase MB, troponins and other proteins. A number of mechanisms have been proposed to explain the initiation and execution of necrosis, including death receptors, ROS and Ca2+ overload. Therefore the most obvious connection between prolonged myocardial ischaemia with or without reperfusion and necrotic cell death is mPTP opening. Cyclophilin D is an important regulator of mPTP [34]. Cells lacking cyclophilin D are resistant to oxidative-stress- and calcium-induced cell death, but are sensitive to apoptotic stimuli [35]. Mice lacking myocardial cyclophilin D are protected from ischaemia/reperfusion-induced cell death in vivo, whereas cyclophilin D-overexpressing mice show mitochondrial swelling and spontaneous cell death [36]. These studies confirm that necrosis is a major form of cardiac myocyte death in ischaemia/reperfusion injury and demonstrate the importance of cyclophilin D in this process. Finally, emerging evidence suggests that a proportion of necrosis is regulated by serial signalling events in a controlled and sequential manner. Several terms have been introduced to describe this form of necrosis, such as programmed necrosis and necroptosis.

Apoptotic cell death can transition to necrosis during oxidative stress by two possible mechanisms. First, the inactivation of caspases due to oxidation of their active-site thiol groups by oxidants or S-nitrosylation can lead to necrosis-like cell death in fatally damaged cells [37]. Secondly, a fall in ATP levels due to the failure of mitochondrial energy production by oxidants can cause apoptosis to change to necrosis [38]. In addition, it was recently found that the pro-apoptotic protein Bnip3 is associated with mitochondrial dysfunction and cell death. Bnip3 is also a potent inducer of autophagy in many cell types, including...
adult cardiac myocytes. Bnip3 overexpression induces selective removal of mitochondria in cardiac myocytes and triggers induction of autophagy independent of Ca^{2+}, ROS generation and mPTP opening [27]. Furthermore, it has been reported recently that AngII (angiotensin II) induces mitochondrial autophagy and biogenesis through mitochondrial ROS in the mouse heart [39].

**Autophagy**

In contrast with necrosis and apoptosis, autophagy is primarily a survival mechanism. Cellular oxidative stress and ROS have been reported to serve as important autophagic stimuli during periods of ischaemia/reperfusion [40]. Autophagic degradation and removal of damaged oxidized proteins in response to low- to-moderate oxidative stress is reportedly beneficial for cells. Conversely, severe oxidative stress and increasing amounts of ROS may activate signalling pathways that lead to autophagy-induced cell death. Whether autophagy promotes cell survival or death depends upon the severity and degree of stress in the cellular environment [41]. During the initial period of ischaemia, the enzyme XO is formed, and substrates for XO (hypoxanthine and xanthine) accumulate. Upon reperfusion, the reintroduction of O_2 leads to XO-mediated superoxide generation due to the presence of xanthine and hypoxanthine [7]. During this period, ATP generation decreases and results in the phosphorylation of AMPK (AMP-activated protein kinase), which leads to autophagosome formation through the inhibition of mTOR (mammalian target of rapamycin) [42]. Meanwhile, ROS damage organelles and cytosolic proteins and cause mitochondrial lipid peroxidation, all of which exacerbate autophagy [43]. Additionally, antioxidant enzymes, such as CAT and SOD, are targeted by autophagosomes. This ultimately leads to the induction of cell death and is thus detrimental to tissue function. Furthermore, AMPK activity decreases during reperfusion, thus increasing autophagic death and up-regulating Beclin-1 [44]. Autophagy has been reported to be involved in cardioprotection against lethal ischaemic injury; thus repetitive ischaemia by coronary artery occlusion or occlusion enhances autophagy and subsequent cardioprotection when compared with a classical ischaemia/reperfusion insult [45]. Aside from the key role that the chemokine MCP-1 plays in cardiac damage following ischaemia, it also mediates autophagy through MCPIP, a novel zinc-finger protein that has transcription-factor-like activity [46]. MCPI stimulates iNOS, translocation of the NADPH oxidase subunit p47phox from the cytoplasm to the membrane, ROS production, induction of endoplasmic reticulum stress markers HSP40 (heat-shock protein 40) and autophagy, as indicated by beclin-1 induction, cleavage of MAP1LC3 (microtubule-associated protein 1 light chain 3) and autophagolysosome formation, and apoptosis respectively [47].

**IMPORTANCE OF THE DURATION OF ISCHAEMIA AND REPERFUSION**

After the onset of ischaemia, ultrastructural changes in myocardium occur rapidly, but they may be considered reversible if reperfusion of the tissue can be effected promptly. However, ischaemia lasting more than 20–30 min results in a state of irreversible tissue injury that is ultimately characterized as necrosis [48]. Furthermore, it is important to allow differentiation of partial ischaemia, which may be reversible, with total obstruction. The factors that influence the onset and extent of irreversible injury include: the size of the area at risk [49], the extent of collateral blood flow or residual flow through the infarct-related artery, the duration of ischaemia and myocardial temperature [50]. It is clear that reperfusion is the unique strategy for tissue viability and the therapies leading to the myocardial revascularization following STEMI (ST-segment elevation MI) (fibrinolysis, PCI and emergency coronary artery bypass grafting) have the primary aim of salvaging viable tissue, within the ischaemic risk zone, and thereby limiting the extent of necrosis.

In relation to the effect of reperfusion, there has been much debate as to whether it would simply accelerate cell death or truly kill cardiomyocytes that would otherwise have survived (in the absence of the deleterious effects of reperfusion). Early studies of apoptosis in experimental infarction using permanent coronary artery occlusion in the rat, without reperfusion, suggested that apoptosis represented the major form of myocyte death [51,52]. Subsequently, the majority of evidence suggests that the number of cells undergoing apoptosis is likely to be relatively low compared with the necrotic cells. However, it is not clear at what stage apoptosis occurs in relation to necrosis or how these two processes are connected. The main mechanism which is associated with initiating apoptosis involves the opening of the mPTP during reperfusion, after ischaemia of sufficient duration, as a key mechanism of cell death, amplifying or accelerating cell death to produce a pattern of reperfusion-induced necrosis [53]. In this view, the magnitude of the fall in ATP levels mainly determines the type of cell death and would be dependent on the duration of the ischaemia. In some models of cardiomyocytes, it has been observed that lengthening ischaemia from 30 to 90 min is consistent with increased ischaemia/reperfusion injury, but for any period of ischaemia tested over 90 % of the cell death proportion was observed during the reperfusion period not during the ischaemic conditions. These results indicate that, although cell injury during ischaemia occurs, most of the loss in cell viability occurs during reperfusion. The inclusion of antioxidants during ischaemia (metal-chelating agents and thiol-donating groups), can substantially protect against this injury, reducing cell death at the final of reperfusion phase, an effect which was maintained at 3 h after ischaemia [54].

The protocol of ischaemic pre-conditioning described by Murry et al. [55] presented an experimental intervention able to limit infarct size in a consistent and reproducible manner. They showed in the anaesthetized dog that four 5-min periods of left anterior descending coronary artery occlusion, interspersed with 5-min reperfusion periods, before a 40-min occlusion of the same artery resulted in a limitation of infarct size. This cardioprotective effect results in the rapid metabolic adaptation of the ischaemic myocardium. There appears to be a critical threshold of ischaemia required to trigger the adaptive mechanism. Therefore it is assumed that not all combinations and durations of ischaemia and reperfusion will trigger the pre-conditioning phenomenon and protect ischaemic myocardium. A pre-conditioning
The overload of O2 to the ischaemic myocardium occurs after the post-reperfusion patients are given vitamin C [64]. The main but these parameters are attenuated to near normal levels when are elevated in the blood of patients with MI after thrombolysis, of the oxidant enzyme XO and MDA (malondialdehyde) levels increased expression in the infarcted sites after MI [63]. Activity and NADPH oxidase in leucocytes [62]. NADPH oxidase has an primary source of ROS during reperfusion, although endothelial creased ROS production (lethal reperfusion). Neutrophils are the ally, procedures designed to recover coronary blood flow, such as increased [61] and further enhances RNS production. Paradoxic- source of NO in the repairing tissue, is also significantly in- the enzyme. Furthermore, macrophage-derived iNOS, a major source of NO in the repairing tissue, is also significantly increased [61] and further enhances RNS production. Paradoxic- ally, procedures designed to recover coronary blood flow, such as PCI and thrombolysis, lead to reperfusion damage via increased ROS production (lethal reperfusion). Neutrophils are the primary source of ROS during reperfusion, although endothelial cells and cardiomyocytes also contribute. Increased ROS product ion is mainly due to XO activation in endothelial cells, mito- chondrial electron transport chain reactions in cardiomyocytes and NADPH oxidase in leucocytes [62]. NADPH oxidase has an increased expression in the infarcted sites after MI [63]. Activity of the oxidant enzyme XO and MDA (malondialdehyde) levels are elevated in the blood of patients with MI after thrombolysis, but these parameters are attenuated to near normal levels when post-reperfusion patients are given vitamin C [64]. The main overload of O2 to the ischaemic myocardium occurs after the occluded artery is opened and myocardial perfusion is restored, whether spontaneously after intravenous thrombolytic therapy or as a consequence of coronary angioplasty. PCI performed dur- ing the first 6 h after the onset of symptoms is recognized as the most useful way to recover coronary flow [65]. The develop- ment of oxidative stress following reperfusion by primary PCI in AMI occurs immediately, as assessed by increased plasma F2-isoprostane levels, which is accompanied by reduced total antioxidant capacity [66]. F2-isoprostanes are products of lipid peroxidation formed by the free-radical-catalysed peroxidation of phospholipid-bound arachidonic acid and released into the circulation. Measurement of F2-isoprostane is a reliable method for assessing oxidative stress in vivo. It is a sensitive parameter in PCI, transiently increasing by approximately 80% in peripheral blood samples during the procedure [67]. Moreover, in coronary sinus blood samples, the concentration of F2-isoprostanes increases by more than 300% after PCI, thus providing direct evidence for enhanced local in vivo oxidative stress in myocardial tissue during reperfusion [68]. Elevated F2-isoprostane levels following PCI have also reported in urine [69]. These findings strongly suggest that coronary angioplasty is associated with an increase ischemia-related oxidative stress.

In addition, it should be expected that oxidative stress leads to oxidation of BH4 (tetrahydrobiopterin), a key redox-active cofactor of eNOS. It is noteworthy that eNOS is only catalytically active in the dimeric form and its ability to bind BH4 is dependent on dimer formation. In fact, BH4 plays a critical role in allowing electron transfer from the prosthetic haem to L-arginine. In the absence of BH4, electron flow from the reductase domain to the oxygenase domain is diverted to O2, leading to a condition known as eNOS uncoupling [70]. Uncoupled eNOS results in the production of superoxide anions rather than NO [71], which further enhances ROS production. This perpetuates a vicious cycle because peroxynitrite, the reaction product of superoxide and NO, leads to further eNOS uncoupling.

Surprisingly, blood from AMI patients had higher antioxid- ant activity of CAT and SOD, but lower levels of non-enzymatic antioxidants, such as vitamin C and vitamin E, when compared with control samples [60]. Similarly, a study of oxidative stress revealed higher antioxidant capacity in patients with AMI treated by PCI, and this effect was due to the release of intracellular anti- oxidants downstream of tissue damage [72]. Levels of antioxidant enzymes were increased in both patients with coronary heart dis- ease and after reperfusion following coronary artery bypass graft surgery [73]. In contrast with these results, decreased SOD and CAT activities were observed in erythrocytes from patients with cardiogenic shock [74] and human blood platelets from patients with AMI who underwent coronary artery bypass graft surgery [75]. In addition, patients with AMI after thrombolysis had re- duced SOD activity [76]. These discrepancies could be due to the different clinical settings of oxidative stress occurrence that give rise to a wide array of oxidative challenge responses. High ROS levels should result in deleterious cellular effects, such as the induction of necrosis, apoptosis or autophagy. It is of interest to note that erythrocytes and platelets cannot induce genomic re- sponses to oxidative challenges, so their antioxidant enzymes are particularly vulnerable to inactivation by ROS-induced structural changes. This could partially explain the discrepancies observed.

Another clinical example of heart ischaemia/reperfusion is post- operative AF (atrial fibrillation), an arrhythmia that is common

**CLINICAL EFFECTS**

Oxidative damage due to myocardial ischaemia/reperfusion events occurs very frequently. Crucial mediators for cardiac dam- age during reperfusion are oxidative stress, inflammation and leucocyte infiltration, all of which are major initiators of ROS. AMI, cardiac surgery with extracorporeal circulation and PCI are clinical models of oxidative stress. AMI is usually initiated by myocardial ischaemia due to coronary artery occlusion. ROS are generated in ischaemic myocardium by a pro-oxidant state that is particularly enhanced after reperfusion; this is exacerbated by decreased non-enzymatic antioxidant defences [60]. The main superoxide source is the NADPH oxidase pathway, with neut-rophils and macrophages as the primary cell types that express the enzyme. Furthermore, macrophage-derived iNOS, a major source of NO in the repairing tissue, is also significantly increased [61] and further enhances RNS production. Paradoxi-cally, procedures designed to recover coronary blood flow, such as PCI and thrombolysis, lead to reperfusion damage via increased ROS production (lethal reperfusion). Neutrophils are the primary source of ROS during reperfusion, although endothelial cells and cardiomyocytes also contribute. Increased ROS product ion is mainly due to XO activation in endothelial cells, mito- chondrial electron transport chain reactions in cardiomyocytes and NADPH oxidase in leucocytes [62]. NADPH oxidase has an increased expression in the infarcted sites after MI [63]. Activity of the oxidant enzyme XO and MDA (malondialdehyde) levels are elevated in the blood of patients with MI after thrombolysis, but these parameters are attenuated to near normal levels when post-reperfusion patients are given vitamin C [64]. The main overload of O2 to the ischaemic myocardium occurs after the occluded artery is opened and myocardial perfusion is restored, whether spontaneously after intravenous thrombolytic therapy or as a consequence of coronary angioplasty. PCI performed dur-
following cardiac surgery with extracorporeal circulation [77]. Increased ROS and inflammation have been suggested to play a key role in the mechanisms of structural and functional damage [78]. A burst of ROS and RNS production occurs shortly after the onset of reperfusion. Consequently, the cardiac tissue is exposed to lipid peroxidation, protein carbonylation and DNA oxidation. In addition, inflammatory cytokines are able to trigger inflammatory processes. These events can severely impair the electrophysiological properties of atrial tissue.

**CARDIOPROTECTION BY ANTIOXIDANTS: VITAMINS C AND E**

Clinical trials designed to study cardioprotection by long-term administration of vitamins C and E have failed to demonstrate beneficial effects (Table 1) [79–86]. Furthermore, concerning ischaemia/reperfusion events, there is major evidence that ROS contribute to myocardial damage in clinical settings including AMI, PCI and post-operative AF. Studies in animal models of AMI suggest that lethal reperfusion accounts for up to 50% of the final myocardial infarct size [17] – damage that is likely to be preventable. Although a number of strategies have been devised to ameliorate lethal reperfusion injury, the beneficial effects in the clinical setting have been disappointing to date. On the basis of the pathogenic role of ROS and RNS, it should be expected that treatments with exogenous antioxidant agents or up-regulation of endogenous antioxidant enzymes or both could protect against reperfusion injury.

**Beneficial effects of antioxidants**

Some studies have suggested that antioxidant agents attenuate left ventricular remodelling following AMI. In patients with AMI who had undergone primary percutaneous transluminal coronary angioplasty, pre-treatment with allopurinol, an XO inhibitor, resulted in effective inhibition of oxygen-derived radical generation during reperfusion therapy and LV (left ventricular) function recovery [87]. More recently, a significant reduction in reperfusion arrhythmias and infarct size were observed after administering the free radical scavenger edaravone to patients with AMI immediately prior to reperfusion [88]. However, other attempts, such as intravenous bolus of SOD [89], had no beneficial effect on patient outcome.
Negative results with vitamin C and E trials, but probable positive acute effects

The oral route has been used in all clinical trials in which vitamin E and C were assayed as long-term cardiovascular protectors. Results of published trials show no benefits of using oral vitamin E or C in patients with cardiovascular history (Table 1). Although CHAOS (Cambridge Heart Antioxidant Study) [79], a-tocopherol treatment significantly reduced the risk cardiovascular death and non-fatal MI, there was a non-significant excess of cardiovascular deaths in this group. Furthermore, that study is methodologically the weaker, with the least number of randomized patients and the shortest follow-up. It is of interest to note that vitamin E was used in a wide range of doses, from 50 mg/day to 800 units daily [79,80], with a great heterogeneity in clinical characteristics of patients included. Some trials enrolled only patients with a history of AMI [80,81], others were a mix of history of risk factors or definitive atherosclerotic disease [82,84–86], and one of them enrolled only patients with traditional cardiovascular risk factors [83]. In turn, long-term exposure to oral doses of vitamin C should not be expected to result in plasma levels consistent with ROS scavenging (see below), a fact seriously affecting its efficacy to protect against AMI injury. In contrast, infusion of high doses of vitamin C, enough to reach plasma levels of 10 mmol/l, prior to reperfusion might significantly reduce cell death caused by the burst of ROS occurring following re-establishment of blood flow by the currently applied therapeutic procedures.

Mechanistic approach

Most investigations on the protective effects of vitamins C and E have merely focused on their antioxidant power. However, these compounds have the potential to influence a broad range of mechanisms underlying human health and disease, as the biological properties of α-tocopherol and ascorbic acid overwhelm their antioxidant effects. Despite the enormous interest in antioxidant vitamins as potential protective agents against the development of human disease, the actual contributions and mechanisms of such compounds remain unclear. Antioxidants, as well as numerous cardioprotective strategies for reducing lethal reperfusion injury, have failed to provide any benefit to patients during reperfusion heart damage [17]. Although the scientific rationale, epidemiological data and retrospective studies have been persuasive, prospective randomized placebo-controlled trials have not verified the actual benefit of antioxidant vitamins in human diseases [90]. There are several possible contributory factors that could explain this discrepancy and the lack of consideration of basic aspects, such as the pharmacokinetic properties of antioxidant vitamins, are discussed below. Consistent with this view, previous attempts to reduce free radical production with oral vitamin C following primary coronary angioplasty for AMI failed to attenuate increased F2-isoprostane production [91]. Jax-Chamiec et al. [92] performed a randomized double-blind placebo-controlled multi-centre study in 800 patients and analysed the combined effects of vitamins C and E through infusion and capsules, but could not demonstrate a major effect of this antioxidant treatment on the clinical outcome, although diabetic patients showed a reduction in 30-day cardiac mortality [93]. It should be noted that the authors acknowledged that the dose of vitamin C used only raised plasma levels to 0.1 mmol/l, a concentration 100 times lower than that required for scavenging superoxide anions. It is of interest to mention that vitamins C and E exert their major beneficial effects behaving as ‘direct antioxidants’. Nevertheless, other agents such as n − 3 PUFA could trigger cell signalling pathways, leading to an enhancement of antioxidant enzymes, thus behaving as ‘indirect antioxidants’ (see below). Both antioxidant pathways could contribute to the reinforcement of the antioxidant defence system and could be considered in a time-course-based design of pharmacological schemes for cardioprotection against ischaemia/reperfusion damage. The beneficial effects of vitamins C and E, as well as those of n − 3 PUFA, will be considered separately.

Vitamin C

Intra-arterial administration of high doses of vitamin C abolished the in vivo effects of superoxide anions on vascular endothelial dysfunction in subjects with essential hypertension [94]. In addition, in vitro studies have also successfully assessed the effects of oxidative stress with and without vitamin C, thus validating the ability of ascorbate to counteract oxidative stress [95]. It should be noted that most human cells have vitamin C transporters coupled with sodium transport at a ratio of 2:1 (sodium/ascorbate), with a Ka5 of 70 μmol/l. Approximately 90% of vitamin C is cytosolic [96]. When vitamin C is administered orally, the relationship between the dose and plasma concentration is sigmoidal. The vitamin C concentration in plasma is tightly controlled, and excess vitamin C is excreted as a function of dose. Doses of 400 mg daily and higher completely saturate the system, producing a steady-state plasma concentration of approximately 80 μmol/l [97]. Unfortunately, this concentration is not enough to scavange superoxide anions. Therefore, in settings accompanied by oxidative stress, such as AMI or PCI following AMI, beneficial effects of orally administered vitamin C should not be expected to prevent oxidative damage. Rather, intravenous infusion should be considered. In fact, superoxide reacts with NO at a rate 105-fold greater than that at which superoxide reacts with ascorbic acid [98]. As a consequence, 10 mmol/l ascorbate is needed to overcome competition with NO for superoxide. Because transporters would be saturated, a high rate of cellular transport is expected at an ascorbate plasma concentration of 10 mmol/l, which is much higher than the Vmax. There are not many studies on vitamin C pharmacokinetics in humans, but they have shown that short-term infusion of high doses of vitamin C induce peak concentrations higher than 20 mmol/l that remain over 10 mmol/l for 3 h [99]. Interestingly, plasma levels up to 60 mmol/l have not resulted in adverse events, except in the case of specific clinical conditions, such as renal impairment or glucose-6-phosphate dehydrogenase deficiency [100], which should be considered within the exclusion criteria established in the protocols designed for using this therapy. Therefore these findings are consistent with the hypothesis that high doses of vitamin C offer an unexplored therapeutic opportunity to counteract myocardial reperfusion damage caused by PCI.

Although oral doses of vitamin C fail to protect the heart against peroxynitrite, beneficial effects other than superoxide
scavenging may be observed when it is administered following AMI. Accordingly, impaired microcirculatory reperfusion improves with vitamin C infusion in patients undergoing elective PCI [101]. Furthermore, SOD levels were reduced significantly in patients subjected to thrombolysis following AMI, whereas XO activity and MDA levels increased significantly. However, oral supplementation of vitamin C in post-reperfusion patients restored these parameters to normal or near-normal levels [64]. These effects could be due to the modulation of ROS production by vitamin C. A major enzymatic source of ROS is NADPH oxidase activity, which is down-regulated by vitamin C. In addition, vitamin C prevents the oxidation of BH4, a cofactor of eNOS that is highly sensitive to oxidation. When BH4 is oxidized, eNOS activity becomes uncoupled, resulting in the production of superoxide rather than NO and enhancing the consequences of oxidative damage [102]. Therefore even oral doses of vitamin C should have beneficial effects on outcome in patients when it is administered before cardiac surgery with extracorporeal circulation. Similarly, post-operative AF patients are also likely to benefit from oral doses of vitamin C. This result is supported by previous studies demonstrating a major reduction in the incidence of post-operative AF in patients subjected to coronary artery bypass who were given 2 g of vitamin C on the day before surgery, followed by 500 mg daily until post-operative day 5 [103]. Another study demonstrated that the combination of ascorbic acid and β-blockers may be more effective in reducing post-operative AF than β-blockers alone [104], and that supplementation of vitamin C reduces the incidence of post-operative AF, the time needed for rhythm restoration and the length of hospital stay [105]. However, available data are few, and additional large-scale clinical trials are necessary to confirm this beneficial effect of vitamin C [106].

It is noteworthy that vitamin C may also abrogate the beneficial effects of ischaemic pre-conditioning. A series of brief sublethal episodes of ischaemia and reperfusion prior to a potentially lethal episode of ischaemia makes the heart more resistant to MI [107]. This pre-conditioning is likely to be abolished due to decrease in the production of ROS, a phenomenon that otherwise could give rise to a survival response by the antioxidant defence system.

**Vitamin E**

Vitamin E, primarily α-tocopherol, is the major peroxyl radical scavenger in biological lipid phases, such as membranes or LDL (low-density lipoprotein) [108,109]. The antioxidant action of vitamin E has been ascribed to its ability to act chemically as a lipid-based free radical chain-breaking molecule, thereby inhibiting lipid peroxidation through its own conversion into an oxidized product, α-tocopheroxyl radical. α-Tocopherol can be restored by reducing α-tocopheroxyl radicals with redox-active reagents such as vitamin C or ubiquinol [110]. The discovery of α-tocopheryl phosphate, a novel and natural water-soluble form of vitamin E, should expand the knowledge of the role of this vitamin in biological systems [111] and facilitate its intravenous administration. In fact, vitamin E oral supplementation hours before acute episodes of oxidative stress may be not effective, but short-term parenteral administration has been shown to enrich vitamin E in endothelial cells [112]. In clinical studies of ischaemia/reperfusion injury, positive effects of a multi-vitamin antioxidant solution, including pre-operative administration of vitamin E, have been described for revascularization of the lower extremities [113], kidney transplantation [114], liver surgery and aortic aneurysm repair [115]. However, similar studies in the myocardium have not been performed. Although it was reported that long-term vitamin E supplements given at a high dose (>400 units/day) may increase all-cause mortality [116], this was not the case for short-term administration of conventional doses required for the prevention of myocardial reperfusion damage.

**Other biochemical effects of vitamins C and E**

Vitamin C at present is considered much more than just an antioxidant [117]. It can diminish ROS production either through stabilization of BH4, thereby avoiding eNOS uncoupling, and can down-regulate NADPH oxidase, the latter a property being shared with vitamin E [118]. In addition, anti-inflammatory effects can be induced by vitamin C due to its ability to inhibit sPLA2 (secretory phospholipase A2) [119]. In turn, vitamin E acts not solely as a ROS scavenger, but also by inhibiting the transcriptional activity of NF-κB [9] or by diminishing ROS production via down-regulating NADPH oxidase. The inhibition of the transcriptional activity of NF-κB should also contribute as an anti-inflammatory effect [10], since NF-κB is able to trigger the expression of pro-inflammatory genes. Although vitamins C and E exert their individual biochemical effects in water or lipid phases respectively, they also can interact with each other at the level of interphases, giving rise to synergetic effects of restoring α-tocopherol from α-tocopheryloxyl radical, which otherwise would behave as a pro-oxidant when it accumulates in lipid environments if vitamin E is given alone.

**n−3 PUFAs**

n−3 PUFAs are commonly found in marine and plant oils, having the first double bond in the third position from the methyl end of the carbon chain. They are considered essential fatty acids, as they are necessary for human health but the body cannot synthesize them. It is important to have a suitable ratio of n−3 and n−6 PUFAs (another essential fatty acid) in the diet. Indeed, n−3 PUFAs are not solely fuels, but are pleiotropic compounds owing to their various biological actions: they play a crucial role in brain function, as well as in normal growth and development, reduce inflammation and may help lower the risk of chronic diseases, such as heart disease, cancer and arthritis, among other effects. n−3 PUFAs help reduce inflammation, whereas most n−6 PUFAs tend to promote it. The role of n−3 PUFAs in cardiovascular disease is well established. Clinical evidence suggests that EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) help reduce risk factors for heart disease, including high cholesterol and high blood pressure. Thus intake of these compounds may protect against acute coronary syndrome [120], retard coronary atherosclerosis, decrease mortality [121] and reduce sudden death subsequent to MI [122].

**n−3 PUFAs in post-operative AF**

Although the mechanism of post-operative AF is based on acute factors, such as inflammation and oxidative stress, the vulnerability of myocardial tissue is dependent on tissue redox status at the
Figure 2 Hypothesis attempting to account for the oxidative damage derived from ischaemia/reperfusion in acute MI and the sites of action of vitamins C and E to abrogate the deleterious effects

NADPH ox, oxidized NAPDH.

PROPOSAL OF NOVEL STRATEGIES FOR PREVENTING HEART ISCHAEMIA/REPERFUSION INJURY

On the basis of the paradigm described above, a hypothesis where an enhanced antioxidant defence system diminishes the vulnerability of myocardial tissue to the oxidative challenges could be suggested. This view would be amenable to therapeutic approaches in some clinical ischaemia/reperfusion settings, including the examples of cardioprotection highlighted below.

Post-operative AF

We have previously provided evidence supporting a potential therapeutic strategy for preventing this specific arrhythmia [129]. This strategy was based on a two-step process, consisting of (i) an oxidative stress stimulus that promotes a survival response of the enzymatic antioxidant system in conjunction with $n-3$ PUFA treatment in patients [126,127]. This controversy probably arises from the specific form of $n-3$ PUFA administered to patients. A 1:2 EPA/DHA ratio, applied in the two studies, showed a decrease in arrhythmia incidence. Unsuccessful studies used a higher EPA/DHA ratio. In agreement with this view, a trend toward a benefit from $n-3$ PUFA supplementation when the EPA/DHA ratio was 1:2 was reported recently [128]. However, more mechanistic studies to ascertain the molecular basis of these effects are still lacking.
anti-arrhythmic effects of n−3 PUFAs, it was recently demonstrated that 4-hydroxyhexenal, a six-carbon aldehyde formed from non-enzymatic peroxidation of these fatty acids, activates the Nrf2 pathway in cardiovascular cell types, leading to increased antioxidant enzyme activity [130], which enhances protection against cytokotoxic cardiac oxidative challenges.

This therapeutic design could enhance the effects of the treatments reported previously that used one of the two components alone. In fact, preliminary data are consistent with this view [131]. Moreover, cumulated evidence supporting this proposal should encourage further trials designed to improve the outcome of patients undergoing cardiac and other surgeries involving a risk of AF, thereby reducing the length of hospital stay and overall cost. In addition, this therapeutic scheme is also suitable for live organ donors, which involves an ischemia/reperfusion event.

Reperfusion damage in PCI following AMI

Previous attempts to reduce infarct size in PCI following AMI have been unsuccessful. This could be due to the high reaction rate constant between NO and superoxide, which leads to a highly peroxidan substance that is not counteracted by feasible plasma concentrations of antioxidant vitamins (Figure 2). Nevertheless, we propose the novel hypothesis that administering large doses of ascorbate, enough to reach at least a plasma concentration of 10 mmol/l during the time of the expected increased ROS levels following reperfusion onset, should protect myocardial tissue from reperfusion-mediated damage.

CONCLUSION AND PERSPECTIVES

There is a considerable amount of evidence for the contribution of oxidative stress to myocardial damage in clinical settings related to ischemia/reperfusion events, such as AMI, PCI or post-operative AF, among others. This metabolic derangement could be abrogated with the antioxidant vitamins C and E. Previous attempts to reduce infarct size in PCI following AMI have been unsuccessful. This could be due to inappropriately low ascorbate doses against the high reaction rate constant between NO and superoxide. This reaction produces peroxynitrite, a highly peroxidan substance, which is not likely to be counteracted by plasma vitamin C concentrations that are achieved with high oral doses. Nevertheless, we propose the novel hypothesis that the infusion of ascorbate, at doses high enough to reach a plasma concentration of at least 10 mmol/l during the time of the expected highest ROS levels following reperfusion onset, should protect myocardial tissue from damage. In addition, post-operative AF could be prevented by lowering reperfusion onset, should protect myocardial tissue from oxidative stress to myocardial damage in clinical settings related to ischaemia/reperfusion events, such as AMI, PCI or post-operative AF, thereby reducing the length of hospital stay and overall cost. In addition, this therapeutic scheme is also suitable for live organ donors, which involves an ischemia/reperfusion event.

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Cardioprotection by antioxidant enhancement


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Received 16 December 2011/25 May 2012; accepted 8 June 2012
Published on the Internet 7 September 2012, doi: 10.1042/CS20110663