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Targeting oxidative stress in disease: promise and limitations of antioxidant therapy

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Abstract | Oxidative stress is a component of many diseases, including atherosclerosis, chronic obstructive pulmonary disease, Alzheimer disease and cancer. Although numerous small molecules evaluated as antioxidants have exhibited therapeutic potential in preclinical studies, clinical trial results have been disappointing. A greater understanding of the mechanisms through which antioxidants act and where and when they are effective may provide a rational approach that leads to greater pharmacological success. Here, we review the relationships between oxidative stress, redox signalling and disease, the mechanisms through which oxidative stress can contribute to pathology, how antioxidant defences work, what limits their effectiveness and how antioxidant defences can be increased through physiological signalling, dietary components and potential pharmaceutical intervention.

Oxidative stress
Imbalance between generation of oxidants and the ability to prevent oxidative damage favouring the latter process.

Redox signalling
Signal transduction in which oxidants act as second messengers.

Antioxidant defence
Prevention or repair of oxidative damage.

Antioxidant enzymes
Strictly, enzymes that remove oxidants; broadly, enzymes that contribute to the prevention or repair of oxidative damage. The broader definition is used in this Review.

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The term 'oxidative stress' was first coined by Helmut Sies[1] as an imbalance between production of oxidants and antioxidant defences that may result in damage to biological systems. Since then, the field of redox biology has evolved from concepts of oxidative stress in pathology to redox signalling in physiology[2–4].

Oxidative stress has been shown to participate in a wide range of diseases including atherosclerosis, chronic obstructive pulmonary disease (COPD), Alzheimer disease and cancer, which has revealed the multiple mechanisms by which oxidants contribute to cellular damage[5]. However, the extent to which oxidative stress participates in the pathology of diseases is quite variable, such that the effectiveness of increasing antioxidant defence may be limited in some diseases.

Oxidative stress involves the chemistry of reactions of so-called reactive species derived from oxygen and nitrogen (BOX 1). Understanding which of these species cause damage to macromolecules helps to provide a rationale for improving therapeutic approaches to antioxidant defence. However, so far, the use of small molecules therapeutically has been disappointing, largely owing to overly optimistic and incorrect assumptions about how antioxidants work[6]. For example, scavenging of hydroxyl radical (•OH) is impractical, but preventing its formation by reducing hydrogen peroxide (H_2O_2) production can provide effective prevention of damage. One of the major misunderstandings in the field of oxidative stress concerns the scavenging of superoxide ($O_2^{\bullet-}$) or H_2O_2 by small molecules, which are also ineffective inside cells. This is because the antioxidant enzymes react thousands to millions of times more rapidly with those oxidants than small molecules do and provide the predominant antioxidant defence[6,7]. However, in extracellular fluids where antioxidant enzymes are absent, scavenging of $O_2^{\bullet-}$ and H_2O_2 (but not •OH) is possible with mimics of superoxide dismutase (SOD) and catalase, as discussed below.

It is essential to recognize the limitations that have led to failures in clinical trials and how antioxidant defences can be effective if one is realistic about where, when and to what extent oxidative stress is part of a disease. Indeed, most antioxidant defence within cells is not provided by either exogenous or endogenous small molecules acting as scavengers, but by antioxidant enzymes using their specific substrates to reduce oxidants. Therefore, the major therapeutic opportunities lie in preventing the production of oxidants that cause direct injury to macromolecules, inhibiting downstream signalling by oxidants that results in signalling for inflammation or cell death, and increasing both antioxidant enzymes and their substrates. Currently, there are clinical trials ongoing for ebselen, a glutathione peroxidase (GPX) mimic, for Meniere disease in phase II (NCT02603081); GC4419, a SOD mimic, for squamous cell cancers in phase I (NCT01921426); and sulforaphane, an activator of the NRF2 transcription factor, for COPD in phase II (NCT01335971), among others.

This article reviews the relationships between oxidative stress, redox signalling and disease and presents an overview of the mechanisms through which oxidative stress can contribute to pathology. We focus on current understanding of the mechanisms mediating

Box 1 | **Mechanisms of oxidative stress**

Both endogenous and exogenous agents cause oxidative stress[276]. The term reactive oxygen species (ROS) encompasses molecules derived from O_2, including superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), ozone and singlet oxygen. The use of ROS, as though it were a chemical entity, leads to many imprecise statements because the chemistries of these species are markedly different.

Production of $O_2^{\bullet-}$ by one-electron reduction of O_2 is primarily through leakage of electrons from the mitochondrial respiratory chain, particularly from ubisemiquinone ($QH^{\bullet-}$)[277] (reaction 1):

$$QH^{\bullet-} + O_2 \leftrightarrow Q + O_2^{\bullet-}$$

and the NADPH oxidases that catalyse reaction 2 (REFS[232,278]):

$$NADPH + O_2 \rightarrow NADP^+ + H^+ + 2O_2^{\bullet-}$$

The NADPH oxidases (NOX4, DuOX1 and DuOX2) and some other flavoprotein enzymes reduce O_2 to H_2O_2, by giving $O_2^{\bullet-}$ a second electron before it leaves their active sites[279].

The predominant source of H_2O_2 is dismutation of $O_2^{\bullet-}$, a fast reaction with a rate constant near $10^5\ M^{-1}\ s^{-1}$ that is accelerated to $2 \times 10^9\ M^{-1}\ s^{-1}$ by superoxide dismutases (reaction 3):

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$$

The rate of H_2O_2 production largely determines whether redox signalling, oxidative stress or no significant oxidation occurs. H_2O_2 is reduced enzymatically by 15 enzymes, including catalase (reaction 4):

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

the five peroxiredoxins that use thioredoxin (a small protein with two crucial cysteines, $Trx(SH)_2$) or the eight glutathione peroxidases and peroxiredoxin 6 that use the tripeptide, glutathione (γ-glutamyl-cysteinyl-glycine, GSH) (reactions 5 and 6):

$$H_2O_2 + 2Trx(SH)_2 \rightarrow TrxS_2 + 2H_2O$$

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

where $TrxS_2$ is thioredoxin disulfide and GSSG is glutathione disulfide.

H_2O_2 does not easily oxidize most molecules but it can react rapidly with transition metals such as iron to produce hydroxyl radical (reaction 7, often referred to as the Fenton reaction)[280]:

$$H_2O_2 + Fe^{2+} \rightarrow OH^- + Fe^{3+} + {}^{\bullet}OH$$

The hydroxyl radical is an extraordinarily strong oxidant that will rapidly oxidize whatever molecule it is next to.

One reaction responsible for oxidative stress is the lipid peroxidation chain reaction that can be initiated by •OH (reactions 8–10):

$$LH + {}^{\bullet}OH \rightarrow L^{\bullet} + H_2O$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$$

where LH is a lipid with allylic hydrogens, which are present in polyunsaturated fatty acids including arachidonic acid.

Superoxide can cause release of iron from iron–sulfur proteins, which can then catalyse reaction 7. The major way that the relatively weak oxidant $O_2^{\bullet-}$ contributes to oxidative stress, however, is as a precursor of H_2O_2 and peroxynitrite ($ONOO^-$), which is formed in reaction 11:

$$O_2^{\bullet-} + {}^{\bullet}NO \rightarrow ONOO^-$$

where •NO is nitric oxide. The danger of producing oxidative stress is not directly from the free radicals, •NO and $O_2^{\bullet-}$, but from the protonated form of peroxynitrite, peroxynitrous acid (ONOOH), a non-radical. Peroxynitrous acid is a very strong oxidant that has the reactivity of the intermediates formed in its decomposition (reaction 12):

$$HNOO^- \leftrightarrow + {}^{\bullet}NO_2 + {}^{\bullet}OH \rightarrow NO_3^- + H^+$$

nitrogen dioxide $\bullet NO_2$ and •OH. $\bullet NO_2$ can abstract hydrogen as does •OH or add to some molecules including the tyrosines in proteins producing nitrotyrosine that may alter function. $ONOO^-$ can also rapidly cause the release of iron from iron–sulfur proteins[11], promoting •OH production from H_2O_2 (reaction 7).

Both $\bullet NO_2$ and •OH are indiscriminate in what they will oxidize, which creates the havoc called oxidative stress. Again, because of their rapid reactions, the best way this can be addressed is prevention of the formation of $\bullet NO_2$ and •OH.

The final oxidants we consider are the hypohalous acids (HOX) that are formed from H_2O_2 in reaction 13, which is catalysed by phagocytic cell myeloperoxidases:

$$H_2O_2 + X^- + H^+ \rightarrow HOX + H_2O$$

where X^- may be Cl^-, Br^- or even SCN^- (REF.[281]). They play a major role in tissue damage associated with phagocyte-mediated inflammation.

antioxidant defences and what limits their effectiveness, and highlight emerging approaches to therapeutically modulate them. Through greater understanding of the mechanisms through which oxidants act and the limitations and potential of antioxidant therapies, a rational approach can be developed that will improve therapeutic intervention.

For the purposes of this Review, we refer to oxidative stress as the situation in which oxidants non-enzymatically damage macromolecules, including proteins, nucleic acids and the lipids that compose cell membranes. This Review focuses only on factors that either prevent production of oxidants or allow their efficient removal. The principal targets are $O_2^{\bullet-}$, H_2O_2 and lipid hydroperoxides. By eliminating these targets, production of the more reactive •OH, peroxynitrite ($ONOO^-$) and the hypohalous acids (HOX) can be prevented. Although $ONOO^-$ production can be limited by inhibiting nitric oxide (•NO) production, because •NO is too important in maintaining normal physiology, the better approach is to limit excessive $O_2^{\bullet-}$ production.

NRF2 transcription factor
Nuclear factor E2-related factor 2, which coordinates both the baseline and stress-inducible activation of a great many antioxidant enzymes.

Roles of oxidative stress in disease

There are two major mechanisms through which oxidative stress contributes to disease. The first involves the production of reactive species during oxidative stress — particularly •OH, $ONOO^-$ and HOCl — that directly oxidize macromolecules, including membrane lipids, structural proteins, enzymes and nucleic acids, leading to aberrant cell function and death. The second mechanism of oxidative stress is aberrant redox signalling (BOX 2). Oxidants, particularly H_2O_2 generated by cells upon physiological stimulation, can act as second messengers[8]. In oxidative stress, non-physiological production of H_2O_2 can cause redox signalling to go awry[4]. Both types of oxidative stress mechanism can occur in a single disease, such as in diabetes, where both advanced glycation products accumulate and aberrant

Antioxidant therapy
Treatment with agents that enhance antioxidant defence.

Pneumonitis
Inflammation of the lungs caused by irritation of lung tissue, disease, infection, radiation therapy or allergy.

Ischaemia–reperfusion
Cessation followed by restoration of blood flow.

Autophagy
A mechanism through which unnecessary or damaged cellular components are degraded.

activation of stress signalling pathways leads to diabetic complications[9]. Also, the increase in H_2O_2 production and iron release from proteins in oxidative stress by $O_2^{\bullet-}$ (REF.[10]) and $ONOO^-$ (REF.[11]) causes a marked elevation in the production of lipid peroxidation products including 4-hydroxy-2-nonenal (HNE), which can also cause aberrant signalling[12].

Oxidative stress has been associated with a wide range of pathologies. On the basis of the contribution of oxidative stress to the aetiology of these pathologies, they have been grouped into two categories below: first, oxidative stress as the primary cause of pathology (including toxicities caused by radiation and paraquat, and in atherosclerosis); second, oxidative stress as the secondary contributor to disease progression (such as in COPD, hypertension and Alzheimer disease). However, as the role of oxidative stress in many diseases is incompletely understood, this categorization is tentative.

Oxidative stress as the primary cause of pathology

Oxidative stress can be a primary factor in toxicity and disease. However, an important caveat is that once damage begins, antioxidant therapy often fails to inhibit the progression of tissue injury as other factors become dominant in the pathology.

Radiation-induced lung injury. Early pneumonitis followed by fibrosis frequently occur as side effects of radiotherapy for lung and oesophageal cancers[13]. When cells are exposed to radiation, homolytic cleavage of H_2O directly generates •OH, which then oxidizes macromolecules and triggers an inflammatory response leading to infiltration of inflammatory cells into the lung (pneumonitis) and cell death. Over a longer period, aberrant redox signalling for the continuous production of cytokines causes accumulation of collagen and lung fibrosis[14]. In addition, higher lipid peroxidation and DNA oxidation (8-hydroxy-2′-deoxyguanosine) has been observed in lungs of radiation-induced lung injury in rats, which can persist for months after radiation exposure[15].

Paraquat poisoning. Oxidative stress is also responsible for the toxicity of the widely used chemical herbicide, paraquat. When ingested, paraquat is actively taken up by alveolar type II cells and leads to pneumonitis and progressive lung fibrosis with poor prognosis. Paraquat also causes injury to other organs including liver and kidney. Long-term exposure to paraquat is associated with Parkinson disease[16]. Paraquat toxicity is initiated by the continuous redox cycling that generates $O_2^{\bullet-}$ (REF.[17]).

Atherosclerosis. In atherosclerosis, plaque builds up in the intimal layer of arteries and over time the arteries narrow, leading to infarction and stroke. Substantial evidence indicates that oxidative stress has a crucial role in the pathogenesis of atherosclerosis. Since the first identification of lipid hydroperoxides in human atherosclerotic aorta[18], many studies have shown an increase in oxidized lipids and other oxidative stress markers in the atherosclerotic lesions. For example, 20% of cholesteryl linoleate (Ch18:2) in freshly isolated human plaque was reported to be oxidized, whereas it was undetectable in normal arteries[19]. In addition, HNE-modified low-density lipoprotein (LDL) was found to be elevated by 50% in plasma of patients with atherosclerosis compared with healthy volunteers[20]. Furthermore, isoprostanes, peroxidation products of arachidonic acid, have been reported to be increased at least fivefold in human atherosclerotic lesions compared with human umbilical veins, and oxidized linoleic acid was detected only in human lesions[21]. Oxidative stress is responsible for the conversion of LDL cholesterol into the atherogenic form of oxidized-LDL (OxLDL), which has a crucial role in initiating and promoting the inflammatory response and recruitment of leukocytes in the lesion site, and contributes to the development of atherosclerosis through activation of smooth muscle cells and reduced •NO bioavailability[22].

Oxidative stress as a secondary contributor to disease progression

In many diseases, oxidative stress occurs secondary to the initiation of pathology by other factors. Examples of this are the oxidative stress caused by increased production of $O_2^{\bullet-}$ or H_2O_2 from NADPH oxidases (NOXs) in the inflammatory response that follows initial tissue injury, and by xanthine oxidase in ischaemia–reperfusion. Oxidative stress can disturb various signalling pathways and affect multiple biological processes through modifying proteins, promoting inflammation, inducing apoptosis, deregulating autophagy, impairing mitochondrial function and many other mechanisms. These effects frequently accelerate pathological progression and exacerbate the symptoms of diseases, as discussed in representative examples below.

Box 2 | **Redox signalling, homeostasis and antioxidant defences**

Redox signalling is dependent on specific interactions of signalling proteins with hydrogen peroxide (H_2O_2) or other electrophiles that act as second messengers. As with oxidative stress, both endogenous and exogenous sources of H_2O_2 or other electrophiles may be involved; however, for redox signalling to be physiological rather than pathological, regulation is essential and requires the involvement of specificity that is not part of oxidative stress. An oxidative challenge, as opposed to oxidative stress, involves the stimulation of redox signalling without any damage, a phenomenon that we have called 'para-hormesis'[101]. A related term is 'oxidative eustress'[3].

Maintaining redox homeostasis is important for cell function. Despite its name, homeostasis does not imply that nothing is changing. Indeed, a balance between oxidants and reductants, including glutathione, thioredoxin and NADPH, which are the substrates for antioxidant enzymes, is essential for maintaining normal physiology[101]. Thus, diseases that involve oxidative stress can be due to disruption of redox homeostasis, with type 2 diabetes mellitus as one example[9]. Adaptive homeostasis, as defined by Kelvin Davies[282], involves elevated antioxidant defences brought about by transient alteration of redox homeostasis and redox signalling. However, redox signalling may also occur under pathological conditions, as oxidative stress can stimulate the same pathways as redox signalling under physiological conditions. The difference in this context is that the signalling will be unregulated and accompanied by nonspecific damage.

The effectiveness of this antioxidant system in maintaining the homeostasis relies upon keeping the generation and removal of superoxide ($O_2^{\bullet-}$), H_2O_2 and nitric oxide (•NO) within a range that does not allow significant production of peroxynitrite ($ONOO^-$) and hydroxyl radical (•OH)[101]. It is not a perfect system as evidenced by a low rate of oxidized proteins that accumulate with age. Regardless, the ability to induce the enzymes that remove $O_2^{\bullet-}$ and H_2O_2 and damaged proteins in what Davies calls 'adaptive homeostasis' provides a major means of enhancing antioxidant defences that will be described elsewhere in this Review[282].

Chronic obstructive pulmonary disease. COPD comprises progressive and irreversible chronic bronchitis and/or emphysema. Cigarette smoking, the main cause of COPD, is an abundant source of oxidants. Oxidative stress can lead to oxidation and inhibition of α1-antitrypsin, thus reducing its ability to inhibit neutrophil elastase, a major factor in the pathogenesis of COPD[23]. In addition, chronic exposure to oxidants in cigarette smoke causes and promotes the inflammatory response and other pathological cascades such as cell death and fibrosis in COPD pathogenesis[14]. The sources of oxidants in COPD are both exogenous (for example, cigarette smoking and air pollution) and endogenous (for example, NOX, mitochondria, inducible nitric oxide synthase (iNOS) and myeloperoxidase)[14]. Increased levels of oxidants and lipid peroxidation products, including 8-isoprostane, have been consistently detected in exhaled breath condensate of patients with COPD compared with healthy controls[24]. In addition, HNE (HNE adducts) levels were found to be significantly elevated by at least 50% in airway and alveolar epithelial cells, endothelial cells and neutrophils in patients with COPD compared with healthy controls[25]; and the urinary level of 8-hydroxydeoxyguanosine (8-OHdG), a marker of DNA oxidation, was significantly elevated in patients with COPD[26]. The level of oxidative stress was inversely correlated with lung function of the patients[25]. Together, these results suggest that oxidative stress occurs both in the lung and systemically in patients with COPD and contributes to disease pathogenesis.

Idiopathic pulmonary fibrosis. The pathology of idiopathic pulmonary fibrosis (IPF) is characterized by diffuse and progressive mesenchymal fibrosis and mild inflammation in the lung with unknown aetiology. Many studies have shown the presence of oxidative stress in IPF. Oxidative stress markers such as H_2O_2, 8-isoprostane, 8-isoprostaglandin-F2α (8-iso-PGF2α) and ethane are significantly increased in the exhaled breath condensate of patients with IPF compared with healthy individuals[27]. In addition, 8-isoprostane is elevated fivefold[28] and oxidized proteins twofold[29] in bronchoalveolar lavage fluid (BALF) of patients with IPF. HNE in lung[30] and 8-isoprostane in blood[31] are also significantly elevated in IPF. The glutathione (GSH) level in epithelial lining fluid and sputum of patients with IPF is fourfold lower than in healthy controls[32], indicating a deficiency of this important component of antioxidant defence in IPF. H_2O_2 production is apparently mainly from NOX4 (REF.[33]) and dysfunctional mitochondria[34], and GSH synthesis is downregulated by TGFβ signalling[35]. Mounting evidence suggests that oxidative stress plays a significant part in IPF, by promoting fibrogenesis through causing apoptosis of alveolar epithelial cells, activating myofibroblasts and inducing an inflammatory response[36]. Besides oxidative stress, IPF pathogenesis involves a number of processes including apoptosis, senescence, epithelial–mesenchymal transition, endothelial–mesenchymal transition, epithelial cell migration, increased production of chemokines, cytokines and growth factors, as well as mitochondrial dysfunction, endoplasmic reticulum stress, hypoxia and inflammation[37]. These mechanisms are interrelated, with oxidative stress representing an important component of the IPF pathogenesis.

Hypertension. Multiple risk factors such as diet, smoking, lifestyle, genetics and comorbidities contribute to hypertension. More than 90% of cases are essential hypertension with unclear cause. At the molecular level, however, oxidative stress is a common feature of this condition. Experimental studies suggest that oxidants are mainly from NOXs in hypertension[38]. Oxidative markers, including H_2O_2 (REF.[39]), glutathione disulfide (GSSG) to GSH ratio, malondialdehyde (a lipid peroxidation product)[40] and 8-isoprostanes, are significantly increased in the plasma of patients with hypertension[41]. H_2O_2 has a role in the development and progression of hypertension, through influencing angiotensin II signalling, NO signalling and other cellular processes[42]. However, a causative role of oxidative stress in hypertension has not yet been established.

Type 2 diabetes mellitus. Patients with type 2 diabetes mellitus display substantial evidence of oxidative stress that results in microvascular and macrovascular complications[43]. Markers of oxidative stress, including OxLDL to LDL ratio[44], 8-OHdG[45], 8-iso-PGF2α[46], protein carbonyls[47] and GSH conjugation to haemoglobin[48], have been reported to be significantly elevated in the plasma of patients with type 2 diabetes mellitus, as have urine 8-OHdG and 8-iso-PGF2α levels[49]. The increased oxidants in type 2 diabetes mellitus arise from dysfunctional mitochondria[50] and NOX1 (REF.[51]) activated by the diabetic abnormalities of hyperglycaemia and dyslipidaemia.

Alzheimer disease. Alzheimer disease is characterized by the progressive accumulation of extracellular amyloid-β plaques and neurofibrillary tangles inside neurons. Several risk factors (age, genetics, sex, trauma and air pollution) for Alzheimer disease have been identified, but the exact cause remains unclear. However, accumulating evidence suggests that oxidative stress may have a crucial role through multiple pathways[52]. Many studies have demonstrated increased oxidative stress in the brain of patients with Alzheimer disease, including increased levels of F2-isoprostane-α in cerebrospinal fluid[53] and frontal and temporal poles[54], acrolein in amygdala and hippocampus/parahippocampal gyrus[55], and HNE in ventricular fluid[56], hippocampus and inferior parietal lobule[57], and cortex[58]. Increased levels of nuclear and mitochondrial DNA oxidation were also found in frontal, parietal and temporal lobes of the brain of patients with Alzheimer disease compared with age-matched control subjects[59]. In addition, protein oxidation in the hippocampus[60] and protein carbonyls in the cerebral cortex[58] were significantly elevated in the brains of patients with Alzheimer disease. Claims have been made that Aβ(1–42)[61], activated microglia[62], iron accumulation[63] and dysfunctional mitochondria contribute to increased oxidant production[64].

Cancer. Through aberrantly altering signalling transduction pathways that damage DNA and exacerbate

inflammation, oxidants are involved in various phases of tumorigenesis, including transformation of normal cells to tumour cells, tumour cell growth, proliferation, invasion, angiogenesis and metastasis[65]. Conversely, oxidative stress can also trigger apoptosis and ferroptosis, and reduce the opportunity for transformation and thereby prevent tumorigenesis[65]. In addition, oxidative stress is the main mechanism of action of radiation (see Radiation-induced lung injury subsection above) and many chemotherapeutic drugs[66]. Therefore, oxidative stress is implicated in almost all phases of cancer. Cancer cells produce more oxidants than normal cells, and therefore cancer cells are exposed to increased oxidative stress in the loci. The increased oxidants in cancer cells are mainly from mitochondria[67], NOX4 (REF.[68]) and 5-lipoxygenase[69]. Oxidants in the loci may also come from normal cells in or surrounding the tumour mass, such as endothelial cells and inflammatory immune cells. The increase in oxidative markers has been observed in various types of cancer. For example, patients with non-small-cell lung cancer have been shown to exhale more H_2O_2 than control individuals[70]. In addition, increased levels of 8-OHdG[71] were detected in breast cancer tissues compared with matched normal tissues, and 8-OHdG was significantly elevated in prostate cancers[72] and lung cancers[73].

Systemic inflammatory response syndrome. Systemic inflammatory response syndrome (SIRS) is a disorder caused by an exaggerated inflammatory response in the whole body to infectious pathogens or non-infectious insults[74]. SIRS involves the release of oxidants and inflammatory cytokines leading to reversible or irreversible end organ dysfunction and even death. Sepsis is a SIRS caused by infection, which shares common features of inflammation and oxidative stress with SIRS caused by non-infectious insults, and is more frequently studied. Plasma F2-isoprostanes[75], HNE[76] and 8-OHdG[77] have been reported to be significantly increased in patients with severe sepsis. In patients with acute respiratory distress syndrome from SIRS, the level of 8-iso-PGF2α is increased in exhaled breath condensate[78] as is nitrotyrosine in BALF[79]. Oxidants in sepsis originate from several sources depending on the tissues and/or cells, and include iNOS (also known as NOS2)[80], NOXs[81], xanthine oxidase[82] and dysfunctional mitochondria[83]. In addition, the levels of antioxidants such as vitamin C[84], vitamin E[85] and GSH[86] are decreased in sepsis.

Ischaemia–reperfusion injury. Although timely reperfusion is essential to avoid irreversible injury from ischaemia (interrupted blood flow), extensive damage to both the local and distant organs can occur through initiation of a systemic inflammatory response. Ischaemia–reperfusion injury (IRI) has a major role in the pathophysiological changes of several critical clinical conditions including heart attack, stroke and organ transplantation. The molecular mechanisms underlying IRI are multifactorial and involve the inflammatory response and oxidative stress. In the ischaemic phase, lack of oxygen and nutrients results in accumulation of hypoxanthine, release of calcium, activation of xanthine oxidase and induction of pro-inflammatory cytokines; and in the reperfusion phase, production of NO, $ONOO^-$, $O_2^{\bullet-}$ and other oxidants is significantly increased from hypoxanthine/xanthine oxidase[87], mitochondria, iNOS (NOS2) and NOXs[88] in endothelial cells, infiltrated neutrophils and local tissue cells[89]. Markers of oxidative stress including urinary 8-iso-PGF2α are elevated in patients with acute myocardial infarction given thrombolytic therapy, when compared with both age-matched, healthy control subjects and patients with stable coronary heart disease[90], and in patients with coronary angioplasty following carotid reperfusion[91]. A study involving 66 individuals with stroke and 132 control subjects showed that plasma and urinary F2-isoprostanes were elevated immediately and up to day 7 after onset of ischaemic stroke[92]. Urinary 8-OHdG was also increased after reperfusion in acute myocardial infarction[93]. It should be noted that most oxidative markers measured in IRI studies were systemic and few studies determined the presence of these markers in the lesion site.

Antioxidant defences and therapeutic implications

To defend against oxidative injury, organisms have evolved defences primarily dependent upon antioxidant enzymes, supply of their substrates and repair of injury. In response to oxidants and other electrophiles, these defences increase and thereby boost the capacity to detoxify oxidants and/or electrophiles and repair oxidative damage. Agents that enhance these defences are the principal strategies underlying antioxidant therapy.

Extensive studies on the induction of antioxidant enzymes have focused on the regulatory mechanisms, the implications in diseases and potential inducers with therapeutic purpose. Although several transcription factors are redox sensitive and are involved in the induction of antioxidant genes (for example, the induction of haem oxygenase 1 (HO1, encoded by *HMOX1*) through activator protein 1 (AP-1)[94] and peroxisome proliferator-activated receptor-γ (PPARγ)[95], and the induction of glutamate–cysteine ligase (GCL)[96] and SOD1 (REF.[97]) through nuclear factor-κB (NF-κB)), the finding with the broadest effect in this area is the induction of antioxidant genes *GCLC*, *GCLM*, *HMOX1*, *NQO1*, *GSTM1*, *GPX4*, *TXN* and *PRDX1* through NRF2 (REFS[98,99]) (BOX 3).

Oxidant species that present immediate danger to the structural integrity and function of cells are •OH, $ONOO^-$ and HOX. However, these oxidants react too rapidly with membrane lipids, proteins and nucleic acids to be effectively scavenged by exogenous small molecules. Unfortunately, many erroneous claims have been made for •OH scavengers. Although oxidative stress involves the generation of •OH, the proposed scavenging of these radicals in biological systems by exogenous molecules is unsound. All organic compounds react with •OH with similar rate constants approaching diffusion limitation. Thus, no compound has more •OH scavenging activity than the thousands of molecules already present in any biological system. To be 50% effective, any compound would have to be present at equal or greater concentration than all of those endogenous molecules.

The only effective strategy preventing damage by •OH is prevention of its formation. Strategies that have the potential to be successful in that endeavour are prevention of the formation of $O_2^{\bullet-}$ and removal of $O_2^{\bullet-}$ and H_2O_2. The removal of $O_2^{\bullet-}$ also prevents the formation of $ONOO^-$, and the removal of H_2O_2 prevents formation of •OH and HOX.

SODs and enzymes that remove H_2O_2 and lipid hydroperoxides form the front line of defence against oxidative stress. However, there are major differences between the extracellular fluids and within cells, which have therapeutic implications. Extracellular SOD (EC-SOD, SOD3) is generally associated with the outer membrane of cells and is not present in all extracellular fluids. SOD mimics are effective in the extracellular fluids where decreased production of the potentially hazardous $ONOO^-$ has the additional advantage of sparing •NO, which participates in vasodilation and other important physiological processes[100]. Although the outer surface of some cells binds to EC-SOD, the additional catalase activity of most SOD mimics also catalyses removal of H_2O_2, which EC-SOD cannot achieve. Intracellular defences include cytosolic SOD1 and mitochondrial matrix SOD2, which remove $O_2^{\bullet-}$, while catalase in peroxisomes (and cardiac mitochondria), GPXs and peroxiredoxins (PRDXs) remove H_2O_2. Some of the GPXs and PRDXs also reduce lipid hydroperoxides, with two of them (PRDX6 and GPX4) being able to reduce phospholipid hydroperoxides. Within cells, scavenging of $O_2^{\bullet-}$ by small molecules is negligible compared with the rate of removal by endogenous SODs, which have rate constants ($\sim 2 \times 10^9\ M^{-1}\ s^{-1}$) that are millions of times higher than those of most other reactions with $O_2^{\bullet-}$. The outer surface of some cells binds to EC-SOD, which also outcompetes any potential $O_2^{\bullet-}$ scavenger. Nonetheless, SOD mimics are useful in extracellular environments that lack significant EC-SOD. SOD produces H_2O_2, which would seem to be not much of a gain in terms of antioxidant defence; however, the removal of $O_2^{\bullet-}$ prevents formation of the more dangerous $ONOO^-$, while simultaneously sparing physiologically important •NO. Compounds with combined SOD and catalase activities have an advantage over SOD alone.

The second line of antioxidant defence includes the synthesis of thioredoxin (TRX), GCL and glutathione synthetase responsible for the synthesis of GSH, glutathione reductase and thioredoxin reductase, which use NADPH to reduce GSSG and $TrxS_2$. It should be noted that both first-line and second-line enzymes also have a role in physiological redox signalling and the maintenance of redox homeostasis, and that total elimination of H_2O_2 would adversely alter cellular function[101]. Scavenging of H_2O_2 and other hydroperoxides by small molecules is negligible compared with removal by the 15 enzymes that reduce H_2O_2 and lipid hydroperoxides and the two enzymes that reduce phospholipid hydroperoxides. Nonetheless, a few mimics of GPX, including ebselen (see below), have rate constants that approach those of the enzymes. In addition, ebselen may also reduce $ONOO^-$. Although GSH is normally in the millimolar range in cells, it can be depleted during oxidative stress. Thus, compounds that increase GSH by either supplying cysteine, which is limiting for GSH synthesis, or are precursors for GSH, increase the effectiveness of endogenous GPXs or GPX mimics. Increasing synthesis of GSH by induction of GCL, the enzyme that kinetically limits GSH synthesis, also offers a therapeutic advantage. Indeed, finding agents that induce GCL through activation of the NRF2 transcription factor has been a major goal for more than two decades.

A third line of antioxidant defence is repair or removal of oxidized macromolecules. This broad area

Box 3 | NRF2–EpRE signalling

Nuclear factor E2-related factor (NRF2) is a member of the 'cap'n'collar' family of bZIP transcription factors (CNC-bZIP). It was first identified as a transcription factor regulating the expression of β-globin by Moi et al. in 1994 (REF. [283]), and soon after was found to be a transcription activator of NQO1 that bound to the antioxidant response element (ARE) in the promoter[284]. Many detailed studies established that NRF2–ARE signalling has a central role in the regulation of antioxidant gene expression[285]. ARE, the *cis* element of NRF2 binding, is more accurately called the electrophile response element (EpRE) as the 'antioxidant' inducers are electrophiles and include hydrogen peroxide (H_2O_2), some components of intermediary metabolism and products derived from dietary polyphenols[6].

NRF2–EpRE signalling regulates the basal and inducible expression of more than 200 genes that encode proteins involved in antioxidant defence, detoxification, apoptosis, DNA repair, removal of oxidized protein by the proteasome, inflammation and other processes[102,286,287]. The role of NRF2 in the induction of antioxidant enzymes and defence against oxidative stress has been verified in cell and non-human animal models with *NRF2* knockout and/or induction. Mounting evidence suggests that deficiency of NRF2 signalling suppresses the induction of target antioxidant enzymes in response to oxidative stress, increases susceptibility to oxidative damage[288] and accelerates the inflammatory response[289], whereas enhancing NRF2 activity increases the expression of antioxidant enzymes and the defence against oxidative stress.

The molecular mechanism and regulation of NRF2 activation in response to oxidative stress has been discussed in many recent articles. Most relevant to therapeutics is the recent review by Cuadrado et al.[99]. Thus, we only briefly describe the regulation of NRF2 (FIG. 3). Under basal conditions, most NRF2 protein binds to Kelch-like ECH-associated protein 1 (KEAP1) and/or β-transducin repeat-containing protein (βTrCP) and is rapidly degraded by 26S proteasome after ubiquitylation. KEAP1 is an adaptor for Cullin 3-containing ubiquitin ligase E3 complex[290], and βTrCP is a substrate receptor for Cul1-based ubiquitin ligase[291]. In response to oxidative stimuli, KEAP1 is oxidatively modified and loses the capacity to present NRF2 for degradation. Simultaneously, oxidative inhibition of glycogen synthase kinase 3β (GSK3β)-mediated NRF2 phosphorylation at the Neh6 domain stops the interaction of NRF2 and βTrCP. NRF2 can also be activated through p62-mediated autophagic degradation of KEAP1 (REF. [292]). With the activation of these pathways, NRF2, both dissociated from KEAP1 and newly synthesized, escapes from degradation and is then translocated into the nucleus where it forms heterodimers with small Maf or Jun family proteins, binds to EpRE in the promoter and increases transcription of target genes. In the nucleus, NRF2 is competitively suppressed by BACH1 (REF. [293]). ChIP-seq assays identified a considerable overlap of BACH1 (in HEK293 cells) and NRF2 (in mouse MEF cells) target genes[294]. Evidence suggests that the suppressive effect of BACH1 on NRF2 signalling may be gene selective. BACH1 inactivation is required for the induction of HO1 but not for that of thioredoxin reductase 1, even though both genes are regulated by NRF2 (REF. [295]). In *Bach1*-knockout mice, fewer than 10% of the upregulated genes are NRF2 target genes[244]. It should be noted that NRF2 regulation is far more complicated than the simplified pathways, as nuclear factor-κB (NF-κB), PKC, p21, BRCA1, HRD1, CRIF1 and microRNAs are involved in regulating NRF2 signalling by acting on NRF2 expression, protein stability, activation and translocation[99].

With more regulators and interaction pathways being identified, NRF2 activity is clearly regulated by a network of signalling pathways allowing it to hold important roles in multiple biological processes and response to multiple circumstances. Some puzzles remain for NRF2 regulation, including how NRF2 is transported in and out of the nucleus, and the dysregulation and ceiling effect of NRF2 induction under some pathophysiological conditions.

Table 1 | **Clinical status of antioxidant enzyme mimics**

Mimic	Antioxidant	Indications	Clinical trial status and refs
NAC	GSH	Paracetamol toxicity, cystic fibrosis, nephropathy and so on	Phase IV (highest; 529 trials in total)[163]
ALT-2074	GPX	Diabetes, coronary artery disease	NCT00491543, phase II[159]
Ebselen	GPX	Meniere disease, bipolar disorder	NCT02603081, phase II[151,152] NCT03013400, phase II[153,245,246]
GC4419	SOD	Squamous cell cancers of the head and neck	NCT01921426, phase I
AEOL-10150	SOD	Non-human animal models of radiation-induced lung injury and inflammation in stroke	Preclinical[247,248]
EUK-8	SOD and catalase	Non-human animal models of sepsis, heart ischaemia–reperfusion, cardiomyopathy, haemorrhage and ALS	Preclinical[137–141]
EUK-134	SOD and catalase	Non-human animal models of ischaemia–reperfusion injury, sepsis and stroke	Preclinical[142,249,143]
EUK-189	SOD and catalase	Non-human animal models of radiation lung fibrosis, cognitive impairment and hyperthermia	Preclinical[144–147]

ALS, amyotrophic lateral sclerosis; GPX, glutathione peroxidase; GSH, glutathione; SOD, superoxide dismutase.

of research is not directly relevant to the present Review; however, the enzymatic systems for removal of oxidized proteins[102], oxidized fatty acid removal and replacement[103], and oxidized DNA removal and repair[104] are induced by oxidants.

Antioxidant therapeutic strategies

Multiple antioxidant therapeutic strategies are being explored, some of which are currently undergoing clinical trials. These include removal of $O_2^{\bullet-}$ before it can react with •NO to form $ONOO^-$ (reaction 11) and removal of H_2O_2 before it can form •OH (reaction 7) or HOX (reaction 13); increasing GSH using precursors; increasing the synthesis of antioxidant enzymes, particularly through NRF2 activation (BOX 3); inhibition of NOXs (reaction 2); mitochondrial antioxidant defence; supplementing dietary antioxidants; and finally, inhibition of aberrant redox signalling (BOX 2). See BOX 1 for reactions.

SOD and SOD–catalase mimics

Several antioxidant enzyme mimics have been and are currently in clinical trials (TABLE 1). SOD is the only enzyme that can eliminate $O_2^{\bullet-}$ in mammalian cells and is a key component in defence against oxidative stress and in preserving •NO. The therapeutic potential of SOD has therefore generated interest since its discovery in 1969 (REF.[105]), and many SOD mimetics have since been developed. These mimetics include the metalloporphyrins, Mn cyclic polyamines, nitroxides, Mn–salen complexes and fullerenes, and their chemical properties have previously been well summarized[106,107].The early studies on SOD mimics primarily focused on metalloporphyrins (that is, MnTM-4-PyP^{5+} and FeTM-4-PyP^{5+})[108–110], and since the establishment of the structure–activity relationship between metal-site redox ability and SOD activity in the late 1990s[111], more porphyrins or porphyrin-related mimics with higher SOD activity have been developed. The protective effects of many of these compounds have been demonstrated in non-human animal studies or even clinical trials. Mimics of SOD and catalase have rate constants several orders of magnitude lower than the enzymes. Thus, when they enter cells, their contribution to cytosolic antioxidant defence is relatively minor. However, SOD and catalase mimics appear to be effective in extracellular spaces where the concentrations of antioxidant enzymes and substrates are very low or absent (FIG. 1). Some of the mimics may also be effective in the mitochondrial matrix, but they can act as pro-oxidants instead of as protectors of mitochondrial function[112].

Although being developed to remove $O_2^{\bullet-}$ specifically, most SOD mimics are not specific and can also reduce other reactive oxygen or nitrogen species such as $ONOO^-$, peroxyl radical, H_2O_2 and $CO_3^{\bullet-}$ (REFS[113,114]). In addition, some SOD mimics, such as Mn porphyrins, Mn(II) cyclic polyamines and M40403, can act as pro-oxidants and react with thiols[112], ascorbate[115] and tetrahydrobiopterin[116], thereby affecting redox-sensitive signalling pathways and cellular transcription[117,118]. Therefore, some protective effects of SOD mimics might be attributable to activities other than mimicking SOD.

SOD itself was first developed as a drug called orgotein in the late 1970s, but it has not been approved for human use[119]. However, several clinical trials based on the anti-inflammatory property of orgotein have been conducted. A double-blind, placebo-controlled study has demonstrated that orgotein can be used safely and effectively to ameliorate or prevent the side effects of radiation therapy in patients with bladder cancer, such as the incidence of radio-induced acute cystitis and rectitis[120,121]. However, in another clinical trial, orgotein showed no beneficial effect on radiation response or the acute radiation reactions, and caused side effects such as marked subcutaneous infiltration and redness at local injection site in some patients[122]. Currently orgotein is used as an anti-inflammatory agent in non-human animals.

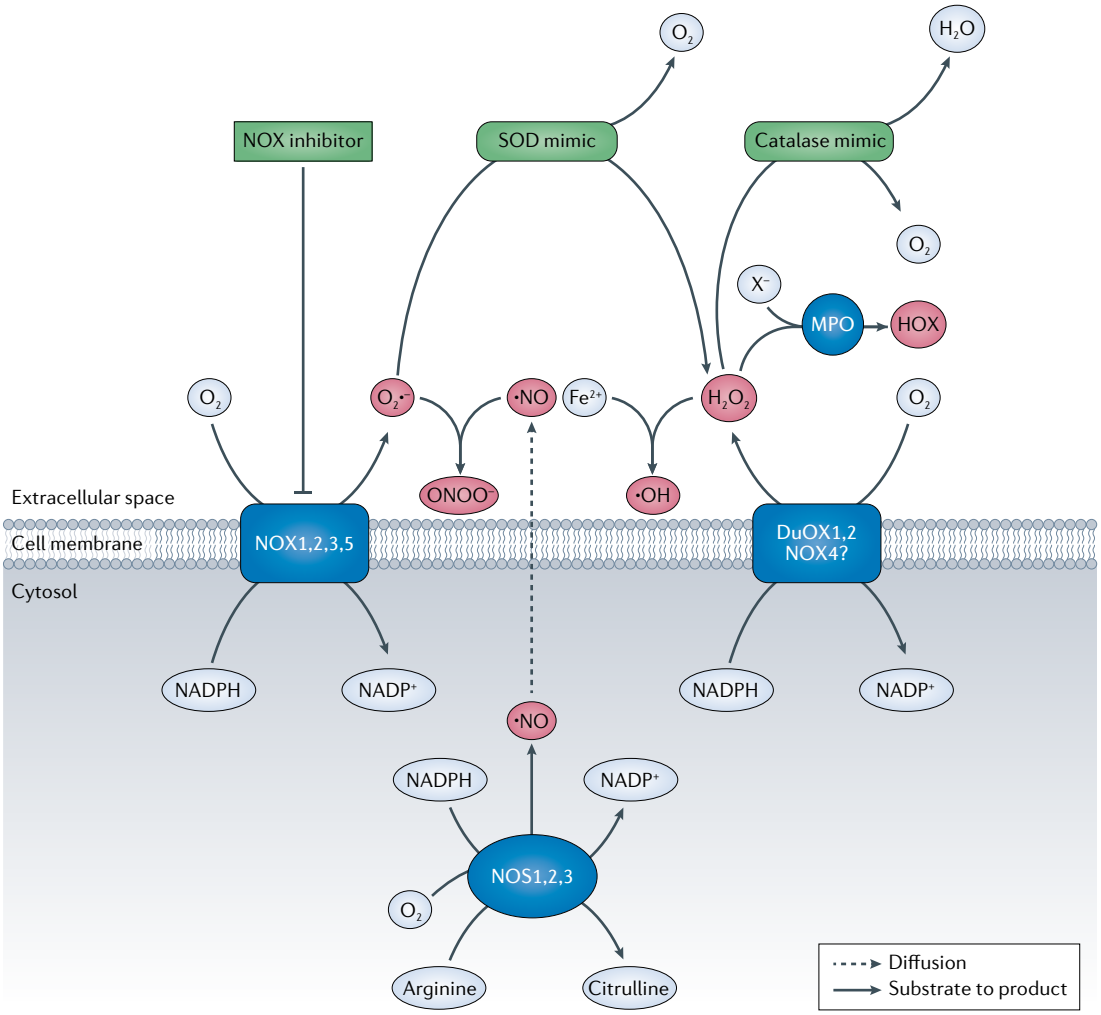

Fig. 1 | **Reactive species in the extracellular space and defences by SOD or catalase mimics and NOX inhibitors.** Plasma membrane NADPH oxidase (NOX) production of superoxide ($O_2^{\bullet-}$) outside cells may be prevented by NOX inhibitors. Dismutation of $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2) is accelerated by superoxide dismutase (SOD) mimics, preventing the formation of peroxynitrite ($ONOO^-$), which spares nitric oxide (•NO). Reduction of H_2O_2 is accelerated by catalase mimics, preventing the formation of hypohalous acids (HOX) by myeloperoxidase (MPO) and hydroxyl radical (•OH) production via the Fenton reaction. Most SOD mimics appear to have catalase activity. Although NOX4, which is primarily in intracellular organelle membranes, has also been found in the plasma membrane, this has only been reported for one cell type[275] and so its extracellular location remains debatable (indicated by the question mark). NOS, nitric oxide synthase.

The best-studied class of SOD mimics is probably the Mn porphyrins. Various Mn porphyrin compounds have been synthesized and evaluated for their $O_2^{\bullet-}$ dismutation activity[114]. Some of them, such as MnTM-2-pYp^{5+} and MnTE-2-pYp^{5+}, showed very high SOD activity. Although whether the underlying mechanism is via SOD-like activity or another action (for example, pro-oxidant activity) remains elusive in some cases, the protective and therapeutic effects of many Mn porphyrins such as MnTE-2-pYp^{5+} and MnTDE-2-ImP^{5+} have been demonstrated in non-human animal models of diseases, including stroke[123], radiation injuries[124], cancers[125,126], diabetes[127] and cardiovascular system damage[128]. These preclinical results suggest the potential of Mn porphyrins in the clinical therapy of diseases in which oxidative stress plays a significant part. Currently, a phase I clinical trial of MnTDE-2-ImP^{5+} in patients with amyotrophic lateral sclerosis showed no toxicity at therapeutic doses[129].

Another promising SOD mimetic is GC4419, a novel, highly stable Mn(II)-containing penta-azamacrocyclic. GC4419 selectively removes superoxide anions without reacting with other oxidants[130]. In vitro, GC4419 significantly enhanced the toxicity of $AscH^-$ to kill cancer cells[131]. In addition, GC4419 has exhibited therapeutic effects in several non-human animal models of inflammation[132], joint disease[133] and myocardial IRI[134]. A recent phase I clinical trial in severe oral mucositis of oropharyngeal cancer with radiation and chemotherapy indicates that the safety of GC4419 in patients is acceptable[135].

Salens, aromatic, substituted ethylenediamine metal complexes, represent an emerging class of SOD mimics. The Mn(III)-containing salen complexes have both

$O_2^{\bullet-}$ and H_2O_2 dismutation activity[136]. Salen compounds are not selective and can also react with other peroxides and $ONOO^-$. The typical representative salens are EUK-8, EUK-134 and EUK-189, which have been shown to be protective in many non-human animal models of human diseases, including sepsis[137], heart ischaemia–reperfusion[138], cardiomyopathy[139], haemorrhage[140] and amyotrophic lateral sclerosis[141] (EUK-8); IRI[142] and stroke[143] (EUK-134); radiation lung fibrosis[144], cognitive impairment[145], diaphragm muscle weakness in monocrotalin-induced pulmonary hypertension[146] and hyperthermia[147] (EUK-189). However, no human clinical trial for salens has yet been reported.

Glutathione peroxidase mimics

A variety of mimics of GPXs have been developed[148]. Among these mimetics, the selenoorganic compound ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) is best known, with its broad specificity for substrates from H_2O_2 and smaller organic hydroperoxides to membrane-bound phospholipid and cholesterol hydroperoxides[149]. Ebselen may also induce phase II detoxification enzymes[150]. In non-human animal studies, ebselen has been shown to reduce oxidative damage[150], prevent the acute loss of outer hair cells and reduce hearing loss[151], and decrease inflammation[152]. Accordingly, several clinical trials have been conducted in diseases including Meniere disease (phase III, NCT04677972), bipolar disorder[153], complete occlusion of the middle cerebral artery[154], delayed neurological deficits after aneurysmal subarachnoid haemorrhage[155] and acute ischaemic stroke[156]. In these studies, oral administration of ebselen was well tolerated, exerted therapeutic effects and displayed favourable bioavailability.

ALT-2074 (BXT-51072) is a newer analogue of ebselen, displaying increased GPX activity and potency. In vitro, ALT-2074 inhibited the inflammatory response in endothelial cells[157], reduced oxidative damage and prevented neuronal death[158], and in a mouse model of heart ischaemia–reperfusion it reduced infarct size[159]. A phase II clinical trial of ALT-2074 (NCT00491543) in diabetes and coronary artery disease has been completed but data are not yet available. Another clinical trial on psoriasis (NCT00782613) was terminated but the reasons for this remain unknown.

Chelation of iron

It has long been recognized that when iron and copper are released from proteins, they can participate in •OH production, and that some chelators enhance that activity while others inhibit it[160]. In principle, using the inhibitory chelators would be an excellent strategy to prevent •OH production; however, as iron is essential for many biological activities, chelation therapy is generally restricted to the prevention of iron overload in patients with sickle cell disease and thalassaemia, who require frequent transfusions[161].

Increasing GSH

Although most cells have a concentration of GSH in the millimolar range, GSH is often significantly decreased by oxidative stress. Thus, approaches to maintaining or replenishing GSH using GSH esters or agents that provide its precursor, cysteine, the limiting amino acid in GSH synthesis, have shown effectiveness in various diseases.

N-*acetylcysteine.* *N*-acetylcysteine (NAC) is one of the most studied antioxidant agents for therapeutic treatment (TABLE 1). It is water soluble and quickly absorbed primarily via the anion exchange protein on the cell membrane[162]. NAC in cells is deacetylated to produce cysteine. Evidence indicates that the antioxidant function of NAC is primarily mediated via replenishing GSH[163]. NAC can also reduce cysteine conjugates in plasma[162]. NAC has been used therapeutically for the treatment of many pathologies, including liver paracetamol (also known as acetaminophen) toxicity[164], cystic fibrosis, where it is delivered through the airways[165] and nephropathy[166]. In non-human animal studies and clinical trials, NAC is being investigated for prevention or treatment of many other diseases and conditions. The results from these studies are conflicting and a consensus has yet to be reached. Failure of NAC to exert a therapeutic effect may be due to oxidative stress being a secondary contributor to the disease being studied.

GSH esters. GSH itself is not effectively transported into most cells, and exogenously administered GSH is rapidly degraded in plasma[167]. Thus, using derivatives of GSH is a strategy for more successful delivery. Ester derivatives of GSH, including monomethyl (GSH-OMe), monoethyl (GSH-MEE), diethyl (GSH-DEE) and isopropyl esters have been synthesized and evaluated for the efficiency of GSH supplementation. In GSH-MEE, the carboxyl group of the glycine residue is esterified (Glu-Cys-Gly-OEt); whereas in GSH-DEE both glutamate and glycine residues are esterified (tEO-Glu-Cys-Gly-OEt). GSH esters are lipophilic, more efficiently transported across the cellular membrane and resistant to degradation by γ-glutamyl transpeptidase in plasma[168]. Once inside cells, GSH esters are rapidly hydrolysed by nonspecific esterases and form GSH. The transport of GSH-DEE into cells seems more efficient than that of the monoester[169], and human cells can rapidly convert the diethyl ester into the monoester, which is hydrolysed into GSH.

The high efficiency of GSH esters to increase cell and/or tissue GSH has been evidenced in many studies in cell and non-human animal models[170–175]. Subcutaneous or intraperitoneal injection of GSH esters into animals was able to increase GSH levels in various tissues including liver[170], kidney[170], spleen, pancreas and heart[176], but not brain[177]. Brain GSH levels can be increased via intracerebroventricular[174] delivery of GSH-MEE[177]. Although oral administration could also increase tissue GSH levels, this is less effective[176].

The relative efficacy of various GSH esters to increase tissue GSH remains unclear owing to limited evidence. Some cell culture-based studies suggest that GSH-DEE is more effective than GSH-MEE in increasing GSH levels[169]. GSH-DEE is metabolized differently in the plasma of non-human animals and humans. In mouse and rat, plasma GSH-DEE is rapidly converted into

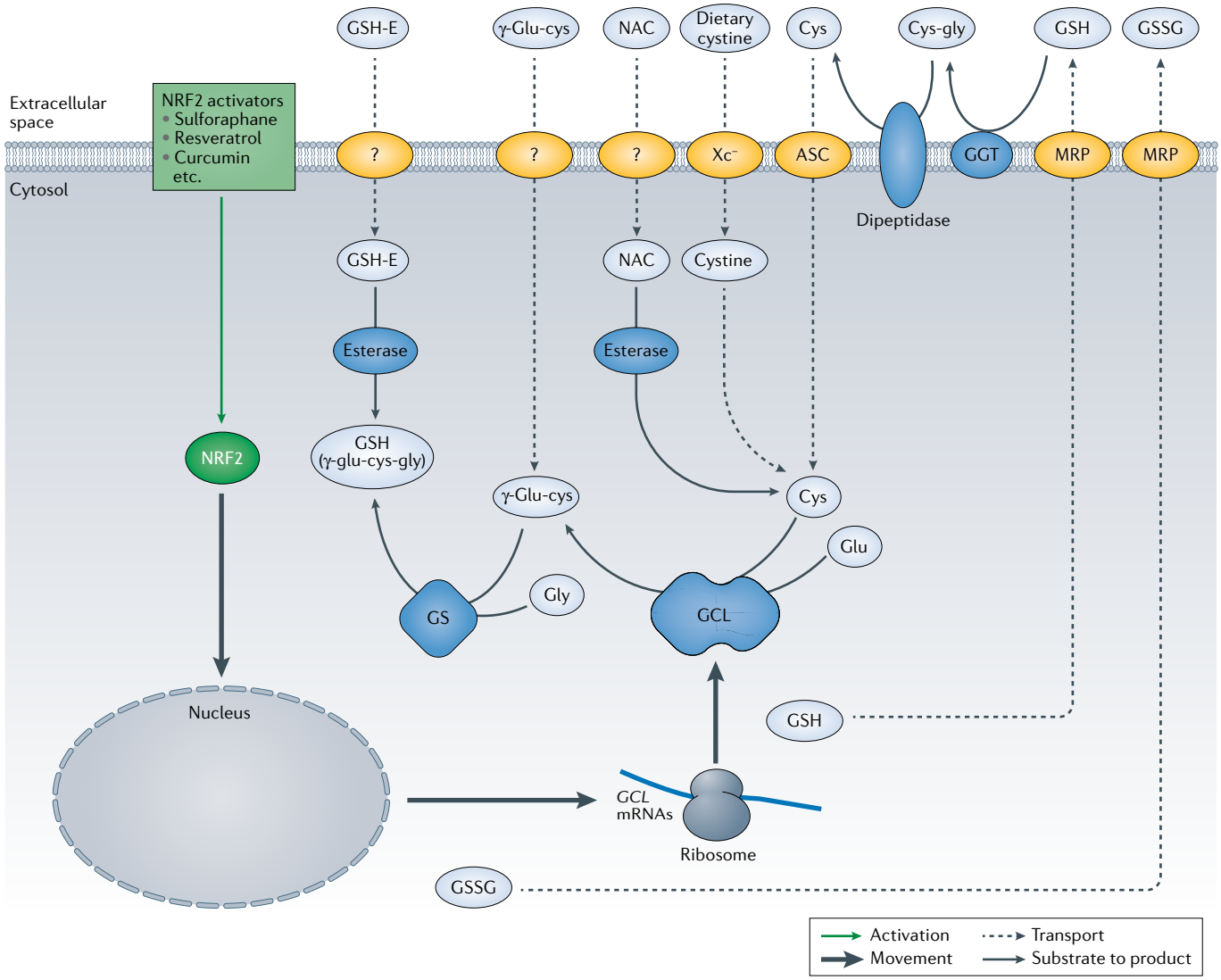

Fig. 2 | **Glutathione metabolism and strategies to increase glutathione.** Glutathione (GSH) is synthesized through reactions catalysed by glutamate–cysteine ligase (GCL) and GSH synthetase (GS), with GCL as the rate-limiting enzyme and cysteine as the rate-limiting substrate. Both reduced GSH and glutathione disulfide (GSSG) are exported from cells through multidrug resistance protein (MRP), and extracellular GSH is sequentially metabolized by membrane-bound γ-glutamyl transpeptidase (GGT) into cysteinylglycine and γ-glutamyl products, and dipeptidase hydrolyses cysteinylglycine to cysteine and glycine. The amino acids are transported back into cells and participate in GSH synthesis. *N*-acetylcysteine (NAC) is deacetylated by esterase action into cysteine, while GSH esters (GSH-E) are directly converted by esterase into GSH. γ-Glutamylcysteine (γ-glu-cys) can bypass GCL, the rate-limiting step for GSH synthesis. Electrophiles cause the activation of NRF2, which regulates the transcription of the two subunits of GCL, and also GS. Some transporters have been identified: ASC, sodium-dependent alanine-serine-cysteine transporter; Xc^-, system cystine/glutamate antiporter. Question marks denote the unidentified transporters/channels for GSH-E, γ-glu-cys and NAC.

GSH-MEE by plasma α-esterase, whereas human (and many other species including hamster, guinea pig, rabbit and sheep) plasma has no α-esterase activity, meaning that GSH-DEE can be transported into tissues more efficiently than GSH-MEE[169]. However, no direct comparison study has been conducted on the relative efficacy of the different GSH esters in clinical settings. Although the reports above suggest that humans have apparently been treated with GSH without adverse effects, and the efficacy of GSH esters to increase GSH levels and alleviate oxidative damage in cells and non-human animals has been demonstrated, no clinical trials have been reported with any GSH ester. FIGURE 2 summarizes the strategies for maintaining GSH in cells.

NRF2 activators

Dysregulation of NRF2 signalling (BOX 3; FIG. 3) is implicated in many oxidative stress-related diseases including cardiovascular diseases[178], neurodegenerative disorders[179] and pulmonary diseases[180]. Therefore, NRF2 activators are regarded as potential agents to induce antioxidant capacity and alleviate pathology. The induction of antioxidant enzymes, particularly through NRF2, is a major way in which antioxidant therapy is being developed. Indeed, when the small molecules such as polyphenols are effective, they act primarily through antioxidant enzyme induction mediated by NRF2 signalling[6]. NRF2 activators comprise five categories, according to their mechanisms of action (FIG. 3): modification

of Kelch-like ECH-associated protein 1 (KEAP1; regulates proteasomal degradation of NRF2), which is inactivated when its sensor cysteines form adducts with electrophiles or when they are oxidized to disulfides; disruption of the interaction between β-transducin repeat-containing protein (βTrCP; ubiquitylates NRF2 for degradation) and NRF2, via oxidative inhibition of the axis of glycogen synthase kinase 3β (GSK3β)–NRF2 phosphorylation at the Neh6 domain–βTrCP; KEAP1 sequestration by p62; de novo synthesis of NRF2 that escapes degradation by inactivated KEAP1 (REF.[181]); and BACH1 inhibitors that reduce NRF2 suppression by BACH1, including agents that inhibit BACH1 translation[182] and promote BACH1 degradation[183].

Extracts from tea, cocoa and many dietary vegetables and fruits including broccoli, broccoli sprouts, grape seeds and turmeric can activate NRF2 signalling and induce antioxidant enzymes[184,185], and some of these are in clinical trials for disease treatment and/or prevention. For example, 11 clinical trials for turmeric extract and 55

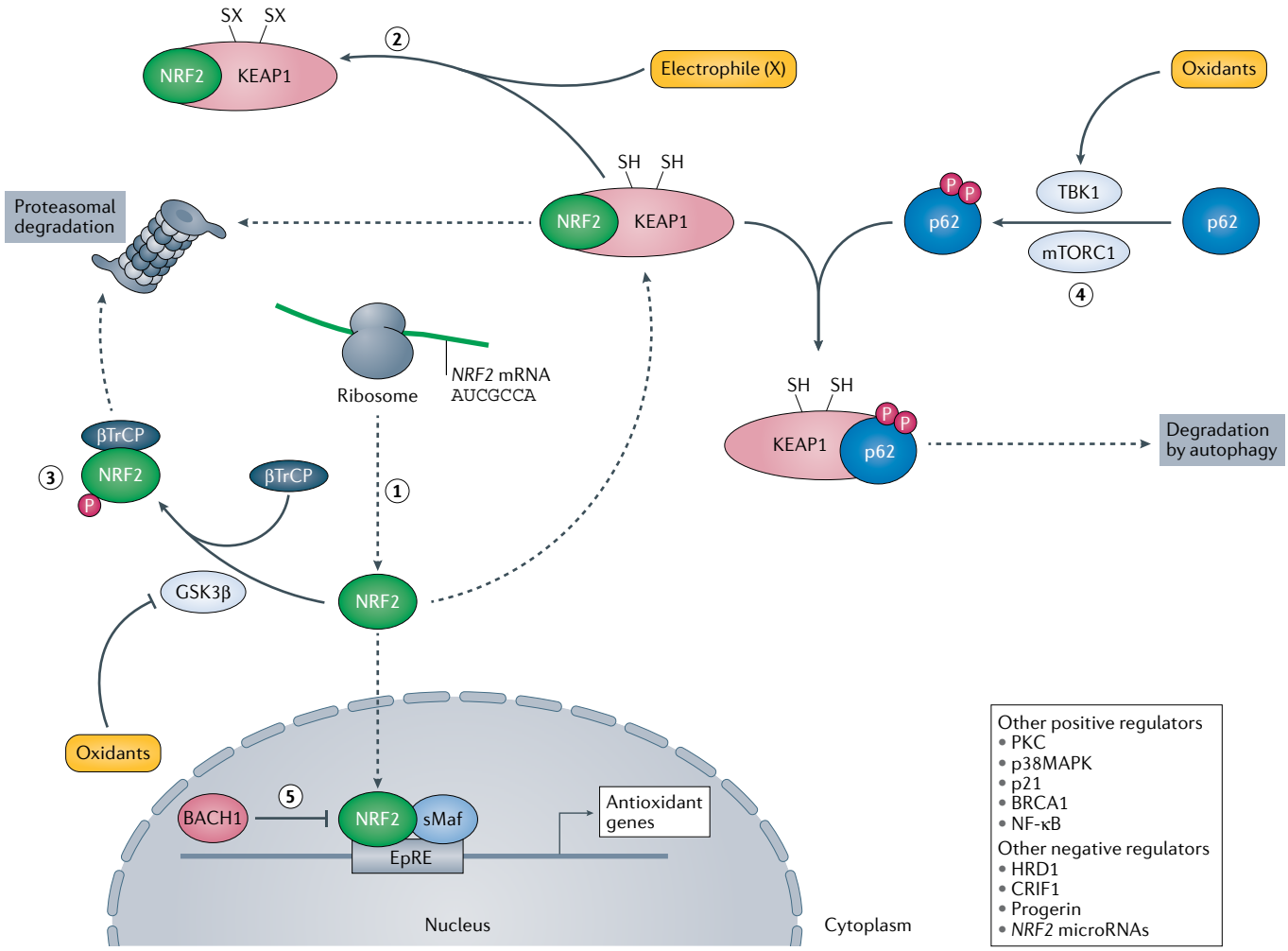

Fig. 3 | **NRF2 signalling pathway and antioxidant therapeutic approaches.** (1) Transcription factor NRF2 is constantly synthesized in cells but its transport to the nucleus remains low under basal conditions. This is due to its degradation through association with Kelch-like ECH-associated protein 1 (KEAP1), which facilitates its degradation by the 26S proteasome. Boosting NRF2 synthesis represents a therapeutic antioxidant approach. (2) Upon exposure to electrophiles, KEAP1 is alkylated and loses its ability to cause degradation of NRF2. Using non-toxic electrophiles to alkylate KEAP1 represents another major therapeutic approach. For KEAP1, SH is the thiol form and SX denotes the adduct formed with the electrophile (X). (3) In a parallel pathway glycogen synthase kinase 3β (GSK3β) phosphorylates NRF2, which with β-transducin repeat-containing protein (βTrCP) is degraded by the proteasome, a process that is inhibited by oxidative inactivation of GSK3β. The interaction of NRF2 and βTrCP is disrupted owing to oxidant-mediated inhibition of GSK3β and the phosphorylation of NRF2 at the Deh6 domain. Inhibiting GSK3β is another potential therapeutic approach to modulate NRF2 signalling. (4) Oxidation-induced KEAP1 degradation also occurs through p62-mediated sequestration of KEAP1 and autophagy, a process initiated by phosphorylation of p62 via TANK-binding kinase 1 (TBK1) and mechanistic target of rapamycin complex 1 (mTORC1). p62 therefore provides another potential therapeutic target. Newly synthesized NRF2 that escapes degradation is translocated into the nucleus where it binds to EpRE sequences in the promoters of antioxidant genes and increases their expression. NRF2 activity is also positively regulated through NRF2 phosphorylation by protein kinase C (PKC)[269] and its interaction with other proteins such as p21 (REF.[270]) and BRCA1 (REF.[271]). (5) In the nucleus, BACH1 negatively regulates NRF2 activity by competing to form heterodimers with small Maf (sMaf) or Jun proteins and binding to the electrophile response element (EpRE)[272–274]. Thus, compounds that inhibit BACH1 offer an alternative therapeutic approach for increasing expression of some NRF2-regulated genes. Other negative regulators of NRF2, which represent potential therapeutic targets include HRD1, CRIF1, progerin and microRNA for *NRF2* (REF.[99]).

Table 2 | **NRF2 activators in clinical trials**

Compound	Indications (proposed action on NRF2)[a]	Trial phase	Clinical trial ID
Sulforaphane	COPD (REF.[197])	II	NCT01335971
	Depressive disorder	II	NCT04246905
	Diabetes mellitus, non-insulin-dependent	II	NCT02801448
	Ageing	II	NCT03126539
	Bladder cancer, bladder tumour, urothelial carcinoma	II	NCT03517995
	Anthracycline-related cardiotoxicity in breast cancer	I/II	NCT03934905
	Autism spectrum disorder (REFS[250,251])	I/II	NCT02561481
	Chronic kidney disease	NA	NCT04608903
Resveratrol	Chronic renal insufficiency (REF.[252])	III	NCT02433925
	Chronic subclinical inflammation, redox status	III	NCT01492114
	Dilated cardiomyopathy	III	NCT01914081
	Friedreich ataxia	II	NCT03933163
	Follicular lymphoma	II	NCT00455416
	Endothelial dysfunction	I	NCT02616822
	Memory	I	NCT01126229
	Chronic kidney diseases, endothelial dysfunction	NA	NCT03597568
	Cystic fibrosis	NA	NCT02690064
	Inflammatory bowel diseases	NA	NCT04513015
	Metabolic syndrome	NA	NCT02219906
	Postmenopausal insulin resistance	NA	NCT03090997
	Type 2 diabetes mellitus	NA	NCT01038089
Quercetin	COVID-19	IV	NCT04468139
	Coronary artery disease progression	III	NCT03943459
	Autism spectrum disorders	II	NCT01847521
	COPD	I/II	NCT03989271
	Chemotherapy-induced oral mucositis	I/II	NCT01732393
	Atrophic oral lichen planus, erosive oral lichen planus (REF.[253])	I	NCT01375101
	Chronic hepatitis C	I	NCT01438320
	Fanconi anaemia	I	NCT01720147
	GERD, acid reflux, reflux	I	NCT02226484
Curcumin	Chronic schizophrenia	IV	NCT02298985
	Major depression	IV	NCT01750359
	Irritable bowel syndrome	IV	NCT00779493
	Periodontitis	IV	NCT04032132
	Periodontitis	IV	NCT04044417
	Leber hereditary optic neuropathy	III	NCT00528151
	Chronic kidney diseases, type 2 diabetes mellitus, polymorphism (REF.[254])	II/III	NCT03262363
	Non-insulin dependent diabetes	II/III	NCT02529969
	Alzheimer disease	II	NCT00099710
	Healthy	II	NCT01489592
	Inflammation, atherosclerosis, cardiovascular disease	II	NCT02998918
	Irritable bowel syndrome	II	NCT01167673
	Multiple sclerosis	II	NCT01514370
	Cervical cancer	II	NCT04294836
	Gulf War syndrome	I/II	NCT02848417

Table 2 (cont.) | **NRF2 activators in clinical trials**

Compound	Indications (proposed action on NRF2)[a]	Trial phase	Clinical trial ID
Curcumin (cont.)	Oral lichen planus	I	NCT03877679
	Chronic kidney diseases	NA	NCT03475017
	Chronic kidney diseases, peritoneal dialysis, haemodialysis	NA	NCT04413266
	Coronary artery disease, oxidative stress, inflammation	NA	NCT04458116
Bardoxolone-methyl (CDDO-Me, RTA402)	Chronic kidney disease, type 2 diabetes mellitus, diabetic nephropathy (REFS[255–257])	II	NCT00811889
	Chronic renal insufficiency, type 2 diabetes mellitus	II	NCT01053936
	Diabetic nephropathy	II	NCT00664027
	Pulmonary arterial hypertension, pulmonary hypertension, interstitial lung disease	II	NCT02036970
	Liver disease	I/II (completed)	NCT00550849
RTA-408 (omaveloxolone)	Friedreich ataxia (REF.[258])	II	NCT02255435
	Mitochondrial myopathies (REFS[259–261])	II	NCT02255422
	Radiation dermatitis	II	NCT02142959
Dimethyl fumarate	Multiple sclerosis (REFS[262–264])	Approved	NCT02683863
Oltipraz	Lung cancer prevention (REF.[265])	I	NCT00006457
CXA-10	Acute kidney injury (nontraumatic) (REF.[266])	I	NCT02127190
Andrographolide	NA (REF.[267])	NA	NA
Ursodiol	NA (REF.[268])	NA	NA
ALKS-8700	NA	NA	NA

COPD, chronic obstructive pulmonary disease; GERD, gastro-oesophageal reflux disease; NA, not available. [a]References describing proposed action on NRF2.

clinical trials for broccoli or broccoli sprout supplement have been completed or are in an active phase for various conditions including COPD, osteoarthritis, joint stiffness and diabetic nephropathy (www.clinicaltrials.gov). Yagishita et al.[186] summarized the current progress on broccoli/broccoli sprout including the formulation, bioavailability, efficacy and doses for clinical trials. In general, some beneficial effects, including a boost of antioxidant capacity, were observed in the clinical trials, but more effort is required to develop and validate biomarkers of pharmacodynamic action in humans. As pointed out above, an increase in antioxidant defence may be limited in disease treatment or prevention if oxidative stress has only a secondary role in the pathology. The underlying mechanism of the antioxidant properties of these dietary supplements, often the coumarins and polyphenols present in vegetables and fruits, relies upon their oxidation to electrophilic quinones that form adducts with KEAP1 cysteines[6].

The effectiveness of many of these NRF2 activators in inducing antioxidant enzymes and in alleviating oxidative damage has been confirmed in non-human animal studies, and there have been significant advances in drug development based on the mechanism of NRF2 activation and antioxidant induction. Several dietary NRF2 activators, including curcumin, sulforaphane and resveratrol, have been developed as daily supplements, while some NRF2 activators are in clinical trials for disease treatment[187]. Selected electrophilic NRF2 activators and the related clinical trials have previously been summarized[187]. It is noted that these NRF2 activators may have multiple functions such as anti-inflammatory effects[188–190], some of which are not dependent on NRF2 activation. TABLE 2 lists the total number of clinical trials of selected dietary NRF2 activators and indicates those that are based on NRF2 activation and/or antioxidant potential. For clarification, it is still possible that some of the agents for which a study of NRF2 activation is not indicated do in fact activate NRF2 even though that was not examined.

Challenges facing therapeutic NRF2 activation. There are several concerns and challenges associated with the therapeutic use of NRF2 activators[191,192]. The first is related to low effective biological concentration, as most NRF2 activators are electrophilic and are metabolized quickly so that their bioavailability in distal organs may be low. However, some evidence suggests that the Michael adducts of nucleophiles (including the cysteines of KEAP1) with some electrophiles, such as cyanoenones, are reversible[193] and this may significantly increase the bioavailability and concentration of these electrophiles in vivo. This concept was demonstrated by a synthesized cyanoenone compound TBE31 that had a 10-h half-life in the blood[194] and markedly increased NRF2 activity in vivo at nanomolar concentrations[195]. It remains unclear whether this reversibility of the covalent adducts also occurs with other electrophiles, especially natural compounds such as sulforaphane and curcumin. In addition, there is controversy regarding the effectiveness of oral sulforaphane to induce antioxidant expression in clinical trials, with both increased antioxidant

expression[196] and no effect[197] being reported. In general, more clinical trial data on NRF2 and antioxidant induction in target organs are needed to further assess the efficacy of these NRF2 activators.

Another key concern is the risk of nonspecific effects. Besides activating NRF2 and inducing antioxidant enzymes, some NRF2 activators may act on other signalling pathways and disrupt related biological processes. For example, sulforaphane can suppress the inflammatory response through inhibition of NF-κB[188] and inflammasome activation[198], and cause cell cycle arrest by inhibiting the PI3K–AKT and MAPK–ERK pathways[199]. Most of these nonspecific effects have been investigated in in vitro cell studies with >10 μM sulforaphane, a concentration that is less likely to be reached in vivo. Understanding the NRF2-independent effects is important in elucidating the mechanism of the beneficial and therapeutic effects, although for most NRF2 activators this has not been thoroughly studied, especially with regard to their in vivo dose dependency.

Another aspect of nonspecificity is that the effect on NRF2 activation and antioxidant induction is not restricted to a specific cell or organ, and may therefore result in systemic side effects. For example, some evidence suggests that although NRF2 activation could prevent the initiation of cancer, it can, however, promote cancer development[200–202]. Cell studies showed that higher NRF2 activity and antioxidant capacity can also contribute to the resistance to chemotherapeutic drugs[203–206], as reviewed by others[207–209]. Current evidence is insufficient to draw a definitive conclusion and more systemic in vivo studies are needed to elucidate the role of NRF2 in promoting carcinogenesis and causing resistance to chemotherapies. If increased NRF2 activity does promote tumour growth and/or increase chemoresistance, the systemic administration of NRF2 activators should be avoided, at least in susceptible subjects including cancer patients under chemotherapy. Other side effects of long-term NRF2 activation are less reported. Several strategies have been proposed to avoid systemic side effects, including the development of non-electrophilic drugs and drugs that only become active in loci that exhibit oxidative stress[192].

NADPH oxidase inhibition

NOXs are important in redox signalling as the source of $O_2^{\bullet-}$ and H_2O_2 and in the killing of microorganisms, but excessive activation of NOXs can result in damage to normal tissue. There are two types of agent that inhibit NOXs, those that inhibit the enzymatic activity and those that prevent the assembly of the NOX2 enzyme, which is a multiprotein complex. Of the first type, diphenyleneiodonium (DPI) is commonly used in research studies but is a nonspecific inhibitor of flavoproteins as well as an inhibitor of iodide transport[210]. Several agents claimed to be NOX inhibitors, including ebselen, CYR5099, apocynin and GKT137831, some of which show promise in non-human animal models and clinical trials, exhibited effects that were not due to NOX inhibition[211]. Nonetheless, the potential value of inhibition of NOX1, NOX2 and NOX4 has been demonstrated in non-human animal models using genetic deletion[212], and a search for low-molecular-weight NOX inhibitors continues.

Small peptides that inhibit the assembly of the NOX complexes have therapeutic potential[213]. Although these small peptides would be more specific to the different NOXs than active site inhibitors, none has advanced to clinical trials. A third potential approach is interference with the synthesis of the components of the NOX complexes; however, this too has not yet reached clinical trials.

Mitochondrial antioxidant defence

Leaks of electrons from the respiratory chain results in the production of $O_2^{\bullet-}$. Although inhibiting $O_2^{\bullet-}$ production by either elevating uncoupling proteins or inhibiting the flow of electrons into the chain is possible, the consequences for ATP production make these approaches difficult. Yet, this strategy has been proposed for preventing hyperglycaemic damage in diabetes[214]. One drug, OP2113, which can be used in humans, has been proposed as a specific inhibitor of complex I $O_2^{\bullet-}$ production that does not interfere with ATP production[215]. However, this agent has not yet been investigated in clinical trials.

As discussed above, increasing SOD2 increases the production of H_2O_2 in mitochondria by pulling reaction 1 ($QH^{\bullet} + O_2 \leftrightarrow Q + O_2^{\bullet-}$) (BOX 1) forward by dismutation of $O_2^{\bullet-}$. Thus, SOD mimics that enter mitochondria would be expected to increase the rate of production of H_2O_2. However, as these agents also possess catalase activity, they appear to add protection[216], likely by preventing formation of $OONO^-$ and protecting iron–sulfur proteins. Ebselen can also enter mitochondria but may produce unexpected toxicity[217].

The large negative inner mitochondrial membrane potential makes it possible to target antioxidants and antioxidant mimics to these organelles by attaching a lipophilic cation to them[218]. This is an area of research that is still under development but basically uses the same principles of antioxidant defence as described in other sections of this Review.

Dietary antioxidants

The most widely used and studied dietary antioxidants are L-ascorbic acid (vitamin C) and α-tocopherol (vitamin E). Other dietary nutrients, including selenium, riboflavin and metals, are essential cofactors for antioxidant enzymes, and their adequate supply is essential for the inducers of these enzymes to reach their most effective levels, but discussion of them here is beyond the scope of this Review. Vitamin C is a water-soluble vitamin that cannot be synthesized by the human body and must be provided as an essential dietary component. Vitamin C is required for the biosynthesis of collagen, protein and several other biological molecules[219]. Vitamin C is also an important antioxidant[220], by providing an electron to neutralize free radicals. Vitamin E, which is lipid soluble, localizes to the plasma membrane and has roles in many biological processes. Almost 100 years after its discovery, the functions and mechanism of action of vitamin E still remain of great interest. Nonetheless, the importance of the antioxidant function of vitamin E has been demonstrated by many studies[221–223], especially under conditions of oxidative stress or deficiency of other antioxidants[223,224]. Vitamin E

reduces peroxyl radicals and forms tocopheroxyl radical, which is subsequently reduced by vitamin C. Thus, vitamin E helps to maintain the integrity of long-chain polyunsaturated fatty acids in the membranes and thereby regulates the bioactivity and signalling related to membrane lipids.

For healthy individuals, sufficient levels of vitamins C and E are provided by normal dietary intake and deficiency rarely occurs. Under some extreme conditions such as malnutrition or imbalanced nutrition and diseases[225,226], however, dietary supplementation of vitamins C and E is necessary. As vitamins C and E function as antioxidants, there has been great interest in investigating their therapeutic potential. Many studies and clinical trials have found that vitamins C and E have beneficial effects in reducing various diseases, many of which likely involve oxidative stress, including cancers, cardiovascular diseases and cataracts[227]. But the evidence is inconsistent, as an almost equal number of studies show no significant effect. It was assumed that both vitamin C and vitamin E have low toxicity and were not believed to cause serious adverse effects at much higher intake than needed for their function as vitamins. However, several non-human animal studies showed that antioxidant supplements, including NAC, vitamin E and the soluble vitamin E analogue Trolox, promoted cancer development and metastasis, for example, lung, melanoma and intestinal tumours in mouse models[228–230]. The potential effect of antioxidants on cancer promotion, including the aforementioned NRF2 activators, raises significant concerns regarding the use of antioxidant supplements, and novel strategies are needed to resolve the double-edged effect of antioxidants.

Inhibition of aberrant redox signalling

In the early years of research in redox biology the emphasis was almost entirely on damage caused by oxidants. Although studies demonstrated that the addition of non-lethal doses of H_2O_2 or other oxidants was able to stimulate signalling pathways, it was not until the mid-1990s that NF-κB activation by endogenous generation of H_2O_2 was first observed[231]. By the late 1990s, Lambeth and coworkers[232] had described the seven-member NOX family and began to implicate them in cell signalling pathways. Redox signalling is now the major focus of the field, although extensive coverage of the topic is beyond the scope of this article. Readers are referred to specific reviews in this area[4,233]. Nonetheless, as described earlier, H_2O_2 is the major second messenger in redox signalling and like other second messengers, dysregulation of its production can result in aberrant signalling[233]. Prevention of dysregulation is tricky because attempts to inhibit the generation of oxidants by NOX proteins or mitochondria, as described in earlier sections, may interfere with physiologically important signalling including the regulation of leukotriene and prostaglandin production, which require a low level of H_2O_2 or lipid hydroperoxides[234].

A more successful approach may be interference with specific redox signalling that is initiated by toxic stimuli. Here, we provide one example to illustrate this approach[235]. Air pollution contains particles of enormously variable composition and includes silicates with iron on their surface. Activation of NF-κB signalling in macrophages by these particles could be inhibited with a SOD and/or catalase mimic, but also by interfering in the signalling pathway initiated by the iron-mediated lipid peroxidation that caused lipid raft disruption and signalling through phosphocholine-specific phospholipase C (PC-PLC) activation. An inhibitor of that enzyme, tricyclodecan-9-yl xanthate (D609), which was unsuccessfully tried as an anticancer agent, stopped particle-induced NF-κB-dependent cytokine production. D609 is an example of an agent that is not an antioxidant but inhibits oxidant-induced aberrant signalling. Interestingly, D609 interferes with the PC-PLC pathway when initiated by endotoxin[236], which does not involve redox signalling. There are countless agents that have similar potential to inhibit aberrant signalling although they are not specific to redox-mediated signalling.

Challenges and limitations in targeting oxidative stress

Oxidative stress is a component of the underlying pathology of many diseases and toxicities, and the antioxidant defences and strategies that have been presented above offer some important opportunities for preventing or reducing pathology. Nonetheless, there are several limitations that challenge our ability to therapeutically apply antioxidant strategies.

Pathological role of oxidative stress

The effectiveness of antioxidant defences is limited by the extent to which oxidative stress plays a role in the pathology. When oxidative stress is a secondary contributor to disease, which is more often the case than it being the primary cause, preventing oxidative stress may not have a major impact on disease progression. Indeed, this is one of the major causes of antioxidants exerting little to no effect on pathology, even when they clearly increase antioxidant defence and decrease markers of oxidative stress. This limitation is perhaps the most significant factor that is often overlooked when considering antioxidant defences in clinical trials. The challenge here is to determine to what extent antioxidant strategies may be developed to ameliorate some symptoms if not the underlying cause of the disease. The commercialization of products containing small molecules that are chemical antioxidants but do not function as such in vivo, will ultimately fail to show significant benefit beyond what the antioxidant enzyme-inducing small molecules present in an adequate diet can achieve. This disappointment will add to the challenge of developing and gaining public acceptance of truly effective therapeutics.

Scavenging by small molecules

The negligible effect of scavenging by small molecules represents a key limitation in antioxidant defence. The claim that an antioxidant is a •OH scavenger is meaningless, as almost all molecules react with •OH at about the same rate. Thus, the only defence against •OH is to prevent its formation, and the most effective way to achieve that is H_2O_2 elimination. For $O_2^{\bullet-}$, scavenging inside the cell is in competition with the already

ubiquitous and high activity of SOD, which catalyses reaction 3 ($2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$) (BOX 1), with a rate constant that is at least 10^5 times higher than most of the reactions of $O_2^{\bullet-}$ except that with •NO[237]. Similarly, the presence of the 15 enzymes that remove H_2O_2 in reactions 4–6 ($2H_2O_2 \rightarrow 2H_2O + O_2$; $H_2O_2 + 2Trx(SH)_2 \rightarrow TrxS_2 + 2H_2O$; $H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$) (BOX 1) would outcompete most agents that are used intracellularly. Thus, kinetic considerations essentially rule out scavenging as an effective antioxidant defence within cells[6]. However, outside cells, SOD and catalase mimics that have relatively high kinetic rate constants compared with non-enzymatic reactions of $O_2^{\bullet-}$ and H_2O_2 may be effective. Although not as efficient as the endogenous SOD and catalase, the rate constants for the mimics are approximately 10^5 times higher than those of most protein cysteines. SOD mimics can accumulate at high concentrations in the mitochondrial matrix by attachment of a lipophilic cationic group and can be effective in that microenvironment[106], where it has been demonstrated that the overexpression of endogenous SOD2 increases H_2O_2 production[238]. However, the long-term effects of the non-physiological increase in mitochondrial SOD activity is unknown.

Vitamin E is the one exception to the limitation of small molecule scavenging by dietary antioxidants because of its relatively rapid rate of reaction with lipid hydroperoxyl radicals as well as its concentration in membranes. Nonetheless, antioxidant therapies that appeared to work in cell culture or in non-human animal models have often failed to achieve significant effects in human trials. A primary reason for this discrepancy is the enormous difference in the ratio of exogenous agents in vitro versus in vivo[6]. In non-human animal models, lab chow is deficient in vitamin E and selenium[239], which sets up a system in which antioxidants work by restoring redox homeostasis, thereby acting more like vitamins preventing a deficiency than like a drug. Interestingly, mito-Q, made by the attachment of a lipophilic cationic group to ubiquinone, can accumulate in mitochondria and act in a similar manner to vitamin E in that domain[240]. However, the long-term effects of the non-physiological increase in ubiquinone is not yet understood.

Achieving effective in vivo concentrations

Another concern is that compounds that induce antioxidant defences may not be able to reach effective concentrations in vivo, although this may be overcome with cyanoenones[194]. When adequate levels of NRF2 activators are supplied by good nutrition, supplemental NRF2 activators would not provide an advantage. In addition, if oxidative stress occurs in patients, NRF2 is usually already activated to a certain degree and the potential for further induction is limited. As a good diet would be expected for patients in clinical trials, and oxidative stress is frequently seen in patients, the lack of an increase in protection may be due to the existing effects of dietary NRF2 inducers and a lower potential for NRF2 activation. Perhaps the use of NRF2 activators should therefore be considered as similar to that of vitamins that are inadequate in the diet of a significant number of individuals and in patients who have difficulty consuming food.

Ageing

As we age, the ability of electrophiles to induce NRF2-dependent expression of antioxidant enzymes declines[241]. Silencing BACH1 reverses this effect in human primary bronchial epithelial cells for some NRF2-regulated genes[242], suggesting that BACH1 inhibition has potential in antioxidant therapy, particularly in older patients. However, as older people exhibit an increased risk of cancer, activating NRF2 in this group may be deleterious. Although NRF2 activation has long been associated with chemoprevention[243], a downside of NRF2 activation is the protection of cancer cells against oxidative damage, which helps cancer progression[200–202]. However, in mice, silencing of BACH1 does not appear to increase p53-driven tumorigenesis[244]. It is hoped that more studies will further clarify the issue of cancer promotion associated with NRF2, and that additional means of increasing antioxidant defences will be found to benefit older people without adverse effects.

Outlook

As oxidative stress is a component of many diseases, the development of effective antioxidant therapies is an important goal. Although using small molecules has been largely disappointing, hope lies in the realization that the rationale underlying their use was based on misconceptions that can be overcome. Increased awareness of the fact that, although the goal of antioxidant defence must be to prevent the formation of •OH and $ONOO^-$ by decreasing their precursors H_2O_2 and $O_2^{\bullet-}$, H_2O_2 is also essential in physiological signalling, will lead to more nuanced approaches to antioxidant defence. In addition, the limitations highlighted in this Review — including consideration of whether oxidative stress plays a primary or secondary role in the pathology, the negligible effect of scavenging by almost all small molecules, difficulty in achieving effective in vivo concentrations and the declining ability to increase NRF2 activation in ageing — must be considered to both avoid unnecessary disappointment and set obtainable goals.

There is promise in agents that scavenge $O_2^{\bullet-}$ and H_2O_2 in intracellular spaces and the mitochondrial matrix. SOD, and SOD–catalase and GPX mimics, appear to be effective, with some agents currently in clinical trials. Maintaining GSH, the substrate for GPXs, can be achieved using precursors including NAC and GSH esters. Indeed, NAC is already in human use for the treatment of some toxicities and diseases, although no clinical trials of GSH esters appear to be currently active. In addition to the mimics of antioxidant enzymes and GSH, another major strategy is increasing the synthesis of the endogenous antioxidant enzymes and de novo synthesis of GSH through NRF2 signalling in cells[99]. We expect that all these approaches will contribute to advancing antioxidant therapeutics and hope that this Review will encourage and inform a rational approach to that worthwhile endeavour.

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