Oxygen radicals, nitric oxide and human inflammatory joint disease

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Interest in the role of free radicals in rheumatoid arthritis (RA) stems from the seminal work of McCord,¹ who noted the decreased viscosity of synovial fluid in RA patients and showed that a similar decrease could be produced by exposing synovial fluid, or solutions of hyaluronic acid, to a system generating superoxide radical, $O_2^{\bullet-}$. McCord's observations led to interest in the use of intra-articular injections of the antioxidant enzyme superoxide dismutase as a treatment in RA. However, the clinical data presented did not convince many rheumatologists^{2,3} and over-enthusiastic interpretations of the data may have led to unwarranted scepticism about the real role of free radicals in RA. Let us review our current knowledge.

Basic definitions

Electrons in atoms and molecules occupy regions of space termed *orbitals*, each of which holds a maximum of two electrons. A *free radical* is any species capable of independent existence that contains one or more unpaired electrons—that is, electrons alone in an orbital. Table 1 gives examples.

Radicals react with other molecules in a number of ways.⁴ If two radicals meet, they can combine their unpaired electrons and join to form a covalent bond (a shared pair of electrons). An important example is the fast reaction of $O_2^{\bullet-}$ with nitric oxide (also a free radical, $NO^{\bullet}$) to form the non-radical peroxynitrite.⁵

A free radical might donate its unpaired electron to another molecule. Thus $O_2^{\bullet-}$ reduces ferric (Fe^{3+}) cytochrome c to ferrous (Fe^{2+}) cytochrome c, a reaction often used to assay $O_2^{\bullet-}$ production by activated phagocytes.⁶

A free radical might take an electron from another molecule, thus oxidising it. For example, $O_2^{\bullet-}$ oxidises ascorbic acid, a process believed to occur in RA:⁷

Left to itself, $O_2^{\bullet-}$ undergoes the *dismutation* reaction:

Hydrogen peroxide, H_2O_2 , is not a free radical (no unpaired electrons are present). The global term *reactive oxygen species* is often used to include the oxygen radicals ($O_2^{\bullet-}$ and

$OH^{\bullet}$) and important non-radical derivatives of oxygen such as H_2O_2 and hypochlorous acid ($HOCl$).

Reactive oxygen species in vivo

The chemical reactivity of oxygen radicals varies (table 1). The most reactive is *hydroxyl radical* ($OH^{\bullet}$), which reacts very fast with almost all molecules in vivo. When $OH^{\bullet}$ is formed, it damages whatever it is generated next to; it cannot migrate within the cell.⁴

NITRIC OXIDE

Whereas $OH^{\bullet}$ is probably always harmful, other (less reactive) free radicals may be useful in vivo. For example, $NO^{\bullet}$ is synthesised from L-arginine by many cell types, including chondrocytes.⁸ However, although human phagocytes can make $NO^{\bullet}$,⁹ it is not yet clear how often they do so in vivo; much of the work with $NO^{\bullet}$ has investigated rats and mice, both of which species have phagocytes that make $NO^{\bullet}$ much more readily.

SUPEROXIDE

Superoxide is produced by phagocytes as a killing mechanism.⁶ Lesser amounts of extracellular $O_2^{\bullet-}$ may be generated, perhaps as an intercellular signal molecule, by several other cell types, including vascular endothelial cells, osteoclasts, chondrocytes, lymphocytes, and fibroblasts.^{10–13} For example, treatment of human fibroblasts with RA synovial fluid causes $O_2^{\bullet-}$ secretion.¹³

In addition to this 'deliberate' $O_2^{\bullet-}$ generation, some $O_2^{\bullet-}$ is produced within cells by mitochondria and endoplasmic reticulum, apparently by the unavoidable 'leakage' of electrons onto oxygen from their correct paths in electron transfer chains and by chemical 'autoxidation' reactions.⁴

HYPOCHLOROUS ACID

Another killing mechanism used by neutrophils (but not macrophages) is the enzyme *myeloperoxidase*,¹⁴ which uses H_2O_2 to oxidise chloride ions into *hypochlorous acid* ($HOCl$), a powerful oxidising and chlorinating agent:

Superoxide-nitric oxide interactions

What happens if both $O_2^{\bullet-}$ and $NO^{\bullet}$ are produced at the same site, for example by

Table 1 Examples of free radicals

Name	Formula	Comments
Hydrogen atom	$\text{H}^\bullet$	The simplest free radical known.
Trichloromethyl	$\text{CCl}_3^\bullet$	A carbon centred radical (the unpaired electron resides on carbon). $\text{CCl}_3^\bullet$ is formed during metabolism of carbon tetrachloride (CCl_4) in the liver and contributes to the toxic effects of this solvent.
Superoxide	$\text{O}_2^{\bullet -}$	An oxygen centred radical. Reacts quickly with a few molecules (such as nitric oxide) but generally not very reactive.
Hydroxyl	$\text{OH}^\bullet$	An oxygen centred radical. The most highly reactive oxygen radical known. When generated in vivo, reacts at its site of formation.
Thiyl	$\text{RS}^\bullet$	General name for a group of radicals with an unpaired electron residing on sulphur. Reactivity varies; often react with oxygen to give damaging oxysulphur radicals.
Peroxy, alkoxyl	$\text{RO}_2^\bullet, \text{RO}^\bullet$	Oxygen centred radicals formed during the breakdown of organic peroxides.
Oxides of nitrogen	$\text{NO}^\bullet, \text{NO}_2^\bullet$	Both are free radicals. $\text{NO}^\bullet$ is formed in vivo from the amino acid L-arginine. $\text{NO}_2^\bullet$ is made when $\text{NO}^\bullet$ reacts with oxygen, and is found in polluted air and smoke from burning organic materials, such as cigarette smoke.

*A superscript dot is used to denote free radical species.

activated macrophages, or when neutrophils adhering to endothelium (a source of $\text{NO}^\bullet$) generate $\text{O}_2^{\bullet -}$? One possibility is that $\text{O}_2^{\bullet -}$ and $\text{NO}^\bullet$ antagonise each other's biological actions. Inappropriate antagonism of $\text{NO}^\bullet$, by excess $\text{O}_2^{\bullet -}$, has been suggested to contribute to impaired endothelium mediated vasodilatation, for example in hypertension.¹⁵

The interaction of $\text{O}_2^{\bullet -}$ and $\text{NO}^\bullet$ can also be dangerous.⁵ The product, peroxynitrite (equation (2)) is not only directly toxic, for example by oxidising methionine and protein -SH groups, but also it breaks down to generate multiple toxic products (figure), including nitrogen dioxide gas ($\text{NO}_2^\bullet$), $\text{OH}^\bullet$ and nitronium ion (NO_2^+).^{5 16} Some of these species will nitrate aromatic amino acids, so that formation of nitroaromatics (especially nitrotyrosine) is thought to be a 'marker' of peroxynitrite generation.^{5 17}

Transition metals and hydrogen peroxide

Many transition metals have variable oxidation numbers: for example iron as Fe^{2+} or Fe^{3+} , and copper as Cu^+ or Cu^{2+} . Changing between these oxidation states transfers single electrons, for example:

Also, for titanium salts:

Thus transition metal ions are good promoters of free radical reactions. For example, copper, iron and titanium ions react with H_2O_2 to form $\text{OH}^\bullet$ radicals:⁴

H_2O_2 is produced in vivo by dismutation of $\text{O}_2^{\bullet -}$ and by several oxidase enzymes, including xanthine oxidase.⁴ Like $\text{O}_2^{\bullet -}$, H_2O_2 can be useful in vivo; for example, it is a substrate for a *thyroid peroxidase* enzyme that helps make thyroid hormones.¹⁸ It can also regulate gene expression, for example by activating the cytoplasmic gene transcription factor NF- κ B.¹⁹ H_2O_2 is very diffusible within and between cells,⁴ but if it comes into contact with transition metal ions, $\text{OH}^\bullet$ will be generated at that site and cause immediate damage.

Antioxidant defences

ENZYMES

Living organisms have evolved multiple antioxidant defence systems. *Superoxide dismutase* (SOD) enzymes remove $\text{O}_2^{\bullet -}$ by accelerating its dismutation by about four orders of magnitude. Human cells have an SOD enzyme containing active site manganese (MnSOD) in mitochondria, whereas cytosol contains a copper and zinc containing SOD (CuZnSOD).⁴ H_2O_2 can be destroyed by catalases, but the most important H_2O_2 removing enzymes in human cells are *glutathione peroxidases*, which remove H_2O_2 by using it to oxidise reduced glutathione (GSH) to oxidised glutathione (GSSG):

METAL ION SEQUESTRATION

Another important antioxidant defence is that iron and copper ions are kept safely protein bound whenever possible, so that $\text{OH}^\bullet$ formation is largely prevented. This is particularly important in extracellular fluids, including synovial fluid, because their levels of antioxidant defence enzymes are low.^{7 20 21}

The value of this sequestration of metal ions is illustrated by an inspection of the severe pathology suffered by patients with metal overload diseases. For example, in patients with iron overload secondary to idiopathic haemochromatosis, transferrin is iron saturated and iron ions 'catalytic' for free radical reactions circulate in the blood.²² Among many other problems, these patients can suffer joint

inflammation,²³ illustrating the well known relationship between chronic inflammation and disordered iron metabolism.²⁴

α-TOCOPHEROL

This fat soluble vitamin functions as a chain breaking antioxidant in membranes and lipoproteins.²⁵ When peroxy radicals are generated during lipid peroxidation (table 1), they abstract hydrogen preferentially from the phenolic –OH group of tocopherol:

This stops peroxy radicals from attacking an adjacent fatty acid side chain or protein and terminates the chain reaction, hence the name *chain breaking antioxidant*. The α-tocopherol radical, tocopherol-O·, is poorly reactive and is widely believed to migrate to the surface of membranes or lipoproteins for conversion back to α-tocopherol by reaction with ascorbic acid.

REPAIR SYSTEMS

The antioxidant defences of the human body are not 100% efficient, so that some free radical damage occurs in the human body and repair systems are needed. Thus cells have enzymes that can repair oxidised DNA, degrade free radical damaged proteins, and remove lipid hydroperoxides from membranes.⁴

Oxidative stress

Oxidative stress is said to result when reactive oxygen species are generated in excess in the human body.²⁶ This can occur if antioxidant concentrations are too low (severe malnutrition, for example, can deplete levels of α-tocopherol and vitamin C) or if free radical formation is increased—for example by the action of certain toxins.⁴

Cells can tolerate mild oxidative stress, and often respond to it by increased synthesis of antioxidant defence enzymes and other protective proteins. However, severe oxidative stress can cause cell injury or even death; oxidative damage to DNA, proteins and lipids can damage or destroy cells. Cell death induced by oxidative stress can occur by necrosis or apoptosis.²⁷ Oxidative stress causes increased intracellular free Ca^{2+} ²⁸ and may release intracellular iron to catalyse OH· generation.²⁹

Reactive oxygen species in RA

GENERAL PRINCIPLES

What is the role played by reactive oxygen species in human disease? Some diseases may be *caused* by oxidative stress, for example the sequelae of overexposure to ionising radiation⁴ and the neurodegeneration produced by chronic α-tocopherol deficiency.³⁰

For most human diseases, however, the oxidative stress is *secondary* to the primary disease process.²⁹ For example, tissue injury recruits and activates neutrophils, which

produce $\text{O}_2^{\cdot-}$, H_2O_2 , HOCl, and possibly $\text{NO}\cdot$; in excess, these cause damage. Tissue injury releases iron and copper ions and haem proteins (haemoglobin and myoglobin), both catalytic for free radical reactions, from their normal intracellular storage sites;^{4,29} it also disrupts electron transport chains in mitochondria and endoplasmic reticulum, so that more electrons leak to oxygen to form $\text{O}_2^{\cdot-}$.³¹ For such secondary oxidative stress, the key question is 'does it contribute significantly to disease pathology?'

THE CASE OF RA

Following the work of McCord,¹ I showed that hyaluronic acid depolymerisation by $\text{O}_2^{\cdot-}$ generating systems in vitro is caused by iron dependent formation of OH· from $\text{O}_2^{\cdot-}$ and H_2O_2 .³² The hydroxyl radical causes random fragmentation of hyaluronate, eventually producing oligosaccharides.³³ How could OH· arise in the RA joint? 'Catalytic' copper ions were not detected in fresh synovial fluid,³⁴ but 'catalytic' iron can be measured by the bleomycin assay in about 40% of synovial fluids aspirated from inflamed RA knee joints,³⁵ and this iron has been directly demonstrated to stimulate lipid peroxidation.³⁶ In addition, aspiration of synovial fluid from some RA patients into a solution of phenylalanine produces a pattern of hydroxylation products characteristic of OH· attack upon the aromatic ring,³⁷ suggesting that constituents of RA synovial fluid can lead to OH· formation.

The catalytic iron could arise by release from dead cells, by H_2O_2 mediated degradation of haemoglobin³⁸ (released by traumatic microbleeding in the joint), or by the action of $\text{O}_2^{\cdot-}$ on synovial fluid ferritin.³⁹ Release of iron upon exposure of synovial fluid to $\text{O}_2^{\cdot-}$, especially at acidic pH, has been demonstrated.⁴⁰ The chemical pattern of damage to hyaluronate in RA synovial fluids (as demonstrated using nuclear magnetic resonance³³) is consistent with OH· attack, though hyaluronate may additionally be secreted as abnormally short chains in RA.⁴¹

Reaction of $\text{O}_2^{\cdot-}$ with $\text{NO}\cdot$ is another potential source of OH·, as the synthesis not only of $\text{O}_2^{\cdot-}$ but also of $\text{NO}\cdot$ ⁴² appears to be increased in RA patients. Demonstration of the presence of nitrotyrosines in patients with active RA⁴³ is consistent with formation of peroxynitrite (equation (1)) in vivo.⁵

A third source of OH· is reaction of $\text{O}_2^{\cdot-}$ with HOCl,⁴⁴ both produced by activated phagocytes:

SOURCES OF OXYGEN DERIVED SPECIES IN RA

The most discussed source is activated phagocytes. Activated neutrophils liberate $\text{O}_2^{\cdot-}$, H_2O_2 , elastase, HOCl, and eicosanoids, and synovial fluid IgG aggregates may activate neutrophils.⁴⁵ Hypochlorous acid and $\text{O}_2^{\cdot-}$ both react with ascorbate, which may help to explain the low levels of ascorbate in RA body fluids.^{7,46} Hypochlorous acid inactivates

α_1 -antiproteinase, an important inhibitor of serpins (such as elastase), and the amount of active α_1 -antiproteinase is decreased in RA;⁴⁷ HOCl also fragments collagen.⁴⁸ The pannus contains many macrophage-like cells, presumably secreting $O_2^{\cdot-}$, H_2O_2 ⁴⁹ and, possibly, NO^* (it is not yet clear if neutrophils or macrophages in the RA joint make NO^*).

It has also been proposed that the inflamed rheumatoid joint, upon movement and rest, undergoes a hypoxia-reperfusion cycle, which may result in free radical generation by several mechanisms.⁵⁰ It is interesting to note that one of these mechanisms is xanthine oxidase,^{51 52} relating back nicely to the original work of McCord.¹

DRUG-DERIVED RADICALS

A few anti-inflammatory drugs may scavenge reactive oxygen species in vivo, but this ability is not widespread.⁵³ Indeed, the reverse can be true: several drugs used in the treatment of RA might themselves be converted into free radicals in vivo. Thus they could suppress the signs of RA whilst aggravating oxidative damage. For example, radicals derived from penicillamine, phenylbutazone, some fenamic acids, and the aminosalicylate component of sulphasalazine, might inactivate α_1 -antiproteinase, deplete ascorbic acid and accelerate lipid peroxidation.⁵³⁻⁵⁵

Consequences of oxidative stress in RA

There is no doubt that oxidative stress occurs in RA patients (table 2).⁵⁶⁻⁷¹ Lunec *et al*⁶⁷ have argued that oxidative damage to IgG generates protein aggregates that can activate neutrophils and set up a 'vicious cycle' of free radical production.

Does oxidative stress contribute to cartilage and bone destruction in RA? The answer is

unclear. Hydroxyl radicals degrade isolated proteoglycans^{1 32 33} and HOCl fragments collagen,⁴⁸ but their effects on intact cartilage are probably limited. However, H_2O_2 is very diffusible and inhibits cartilage proteoglycan synthesis,⁷² for example by interfering with ATP synthesis.⁷³ Indeed, intra-articular injection of H_2O_2 generating systems causes severe joint damage in animals.⁷⁴ Hence inhibition of cartilage repair systems could aggravate the effects of proteolytic and free radical mediated cartilage degradation. HOCl can also activate latent forms of neutrophil collagenases and gelatinase, though the extent to which this happens in vivo is uncertain.⁷⁵ Chondrocytes are damaged by H_2O_2 ,⁷⁶ and it has been suggested that low concentrations of H_2O_2 , $O_2^{\cdot-}$, or both, accelerate bone resorption by osteoclasts,^{77 78} whereas NO^* inhibits it.⁷⁹ In addition, ascorbate is essential for cartilage function⁸⁰ and the low concentrations found in RA synovial fluid might impair cartilage metabolism. The current interest in the role of tumour necrosis factor α (TNF α) in RA⁸¹ may relate to observations that TNF α causes oxidative stress.^{82 83}

In summary, we do not as yet know the exact contribution made by reactive oxygen species to joint damage in RA. The development of improved assays of oxidative damage that are applicable to humans²⁹ should help to address this point and allow a rational selection of antioxidants for possible therapeutic application.⁸⁴

I thank the Arthritis and Rheumatism Council for research support.

Table 2 Evidence consistent with oxidative stress in rheumatoid disease

Observation	Reference	Comment
Increased lipid peroxidation products in serum and synovial fluid	56	Decreased α -tocopherol (per unit lipid) in synovial fluid ⁵⁷ is consistent with increased lipid peroxidation, as are reports of 'foam cells' containing oxidised low density lipoprotein in rheumatoid synovium ⁵⁸ and increased levels of 4-hydroxy-2-nonenal, a cytotoxic product generated by the decomposition of lipid peroxides, in RA. ⁵⁹
Depletion of ascorbate in serum and synovial fluid	See text	Presumably results from oxidation of ascorbate during its antioxidant action. Activated neutrophils also take up oxidised ascorbate rapidly. ⁶⁰
Increased exhalation of pentane	61	A putative endproduct of lipid peroxidation, ⁴ although its validity as an assay is debated. ⁶²
Increased concentrations of uric acid oxidation products	63	Products measured appear to be endproducts of free radical attack upon uric acid. ^{63 64}
Formation of 2,3-dihydroxybenzoate (2,3-DHB) from salicylate in increased amounts	65	2,3-DHB appears to be a product of attack of OH^* upon salicylate in patients taking aspirin. ⁶⁶
Degradation of hyaluronic acid by free radical mechanisms	—	See text.
Formation of 'fluorescent' proteins	67,68	Fluorescence probably caused by oxidative damage to amino acid residues in proteins.
Increased steady state levels (in cellular DNA) and increased urinary excretion of 8-hydroxy-deoxyguanosine (8OHdG)	69,70	8OHdG is a major product of oxidative damage to DNA. ⁴
Increased levels of 'protein carbonyls' in synovial fluid	71	Protein carbonyls are an endproduct of oxidative damage to proteins.

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