Review
Oxidation in rheumatoid arthritis

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Abstract

Oxygen metabolism has an important role in the pathogenesis of rheumatoid arthritis. Reactive oxygen species (ROS) produced in the course of cellular oxidative phosphorylation, and by activated phagocytic cells during oxidative bursts, exceed the physiological buffering capacity and result in oxidative stress. The excessive production of ROS can damage protein, lipids, nucleic acids, and matrix components. They also serve as important intracellular signaling molecules that amplify the synovial inflammatory–proliferative response. Repetitive cycles of hypoxia and reoxygenation associated with changes in synovial perfusion are postulated to activate hypoxia-inducible factor-1α and nuclear factor-κB, two key transcription factors that are regulated by changes in cellular oxygenation and cytokine stimulation, and that in turn orchestrate the expression of a spectrum of genes critical to the persistence of synovitis. An understanding of the complex interactions involved in these pathways might allow the development of novel therapeutic strategies for rheumatoid arthritis.

Keywords: hypoxia, oxidation, rheumatoid arthritis, synovitis

Introduction

Molecular oxygen is essential for the survival of all aerobic organisms. Aerobic energy generation is dependent on oxidative phosphorylation, a process by which the oxidoreduction energy of mitochondrial electron transport is converted to the high-energy phosphate bond of ATP. In this multi-step enzymatic process, oxygen serves as the final electron acceptor for cytochrome *c* oxidase, the terminal component of the mitochondrial enzymatic complex that catalyzes the four-electron reduction of O_2 to H_2O. A byproduct of this process is the production of partly reduced oxygen metabolites that are highly reactive and that leak out of the mitochondria and react rapidly with other molecules. In turn, reactive nitrogen species, sulfur-centered radicals, and other reactive species are generated by interactions with these molecules. Reactive oxygen species (ROS) participate in several physiological functions, and form an integral part of the organism's defense against invading microbial agents.

Because of their potentially damaging effects, several antioxidant mechanisms have evolved to protect cells and organisms from damage by excessive amounts of these highly reactive mediators. Oxidative stress is a term that is used to describe situations in which the organism's production of oxidants exceeds the capacity to neutralize them. The result can be damage to cell membranes, lipids, nucleic acids, proteins, and constituents of the extracellular matrix such as proteoglycans and collagens.

Extended periods of hypoxia, or brief periods of complete anoxia, invariably lead to death. In contrast, cellular hypoxia occurs frequently, both physiologically and pathologically, and serves as a potent stimulus for changes in gene transcription, translation, and several post-translational protein modifications that serve to rapidly adapt cells and tissues to this stimulus. Oxygen levels vary considerably in different tissues – and even in different areas of a single tissue – and depend on a complex interaction of

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AGE = advanced glycation endproducts; HIF-1α = hypoxia-inducible factor-1α; LDL = low-density-lipid proteins; MAP = mitogen-activated protein; MMR = mismatch repair; mtDNA = mitochondrial DNA; NF-κB = nuclear factor-κB; PHD = prolyl hydroxylase; PI-3K = phosphoinositide 3-kinase; RA = rheumatoid arthritis; RNS = reactive nitrogen species; ROS = reactive oxygen species; SOD = superoxide dismutase; TGF = transforming growth factor; TNF = tumor necrosis factor; VEGF = vascular endothelial growth factor; VHL = von Hippel–Landau tumor suppressor factor.

physiological variables, particularly the balance between the vascular supply and the metabolic demands of the tissue. Hypoxia serves as a particularly potent stimulus for angiogenesis in most tissues.

In this review we explore the role of oxidative stress and hypoxia in the pathogenesis of rheumatoid arthritis (RA), a prototypical chronic inflammatory disorder, focusing on recent developments in this area, and highlighting mechanisms that can potentially be exploited therapeutically. An understanding of these processes in the context of RA has been greatly aided by knowledge gained in the areas of cancer and cardiovascular biology.

ROS in health and disease
Generation of ROS
Phagocytic cells such as macrophages and neutrophils, on activation, undergo an oxidative burst that produces highly toxic ROS that are designed to kill the invading pathogens (reviewed in [1,2]). This oxidative burst is mediated by the NADPH oxidase system, and results in a marked increase in oxygen consumption and the production of superoxide ($O_2^{-\bullet}$). NADPH is composed of several subunits that assemble at the plasma membrane and fuse with intracellular phagocytic vesicles or the outer membrane. This allows the concentrated release of oxidants formed subsequently. Superoxide is converted to hydrogen peroxide (H_2O_2) either spontaneously or more rapidly when catalyzed by superoxide dismutatase, an enzyme that occurs in two isoforms, one of which is inducible by inflammatory cytokines such as tumor necrosis factor-α (TNF-α).

In the presence of ferrous ions (Fe^{2+}) and other transition metals, hydrogen peroxide and superoxide are converted via the Fenton reaction to highly reactive, aqueous soluble hydroxyl radicals (OH$^\bullet$) that are probably responsible for much of the cell toxicity associated with ROS. Additionally, the neutrophil-associated enzyme myeloperoxidase can oxidize halides such as chloride (Cl$^-$) and convert hydrogen peroxide into hypochlorous acid (HOCl), which then can interact with amino acids to form chloramines. Similar reactions can occur with other halides such as bromide and iodide. Further reaction of hydrogen peroxide with hypochlorous acid produces singlet oxygen, another highly reactive and damaging radical. Reactions of hypochlorous acid with amino acids lead to aldehyde production. Superoxide can also react with nitric oxide (NO), synthesized from the deimination of L-arginine by nitric oxide synthase (NOS), and produce the highly reactive peroxynitrite radical (ONOO$^-$). These reactions are summarized in Table 1.
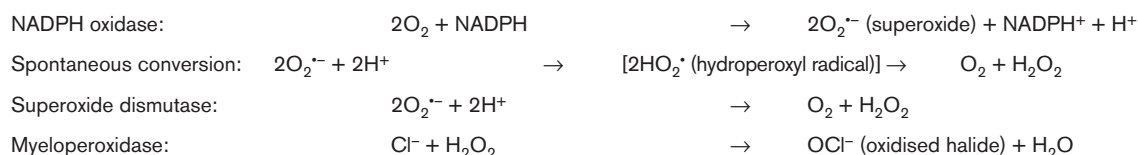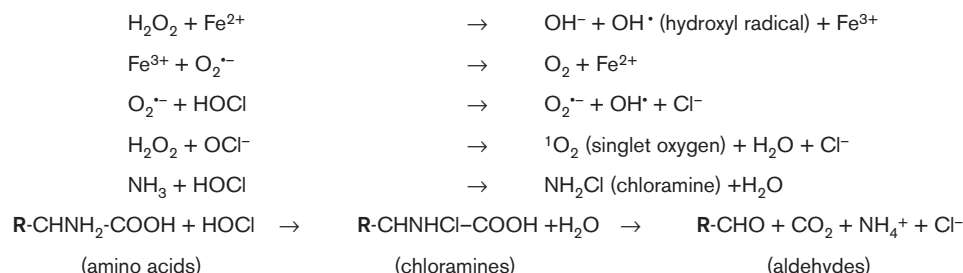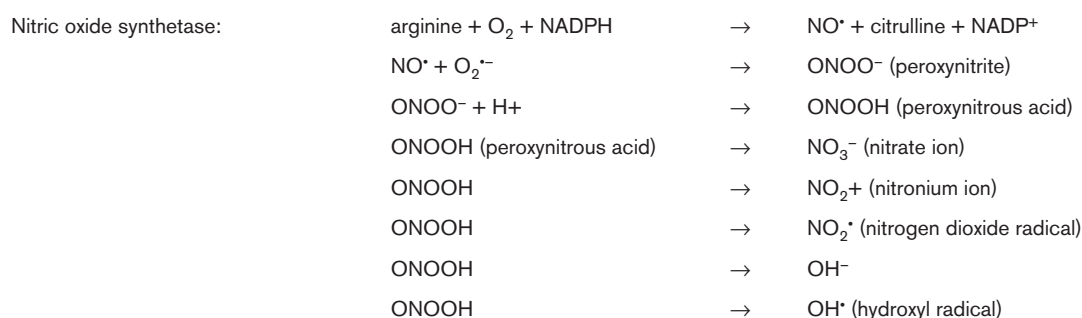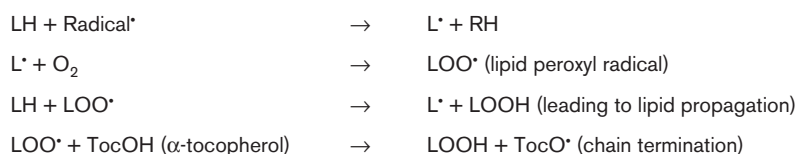
Physiological roles for ROS
ROS are produced during normal aerobic cell metabolism, have important physiological roles in maintaining cell redox status, and are required for normal cellular metabolism including intracellular signaling pathways and the activity of transcription factors such as NF-κB, activator protein 1 and hypoxia-inducible factor-1α (HIF-1α) (see below). In addition, ROS produced by phagocytes also seem to have important physiological roles in priming the immune system. A functional mutation of a component of the NADPH oxidase complex, *Ncf1*, produces a lower oxidative burst and enhanced arthritis susceptibility and severity in murine pristane-induced arthritis [3,4]. Activation of the NADPH complex by vitamin E ameliorated arthritis when given before arthritis induction, indicating that the *Ncf1* functional polymorphism is involved at the immune priming stage of disease. The authors of those papers propose that the physiological production of ROS by phagocytes in response to antigen affects T cell–antigen interactions and possibly induces apoptosis of autoreactive arthritogenic T cells, thereby preventing autoimmune responses. In humans, *Ncf1* is redundant and a complete loss of function is associated with chronic granulomatous disease that has increased susceptibility to microbial infections. The associations of *Ncf1* with other experimental autoimmune conditions suggest that polymorphisms in the *Ncf1* gene might be important for autoimmunity in general [5].

Oxidant defense mechanisms
Several defense mechanisms have evolved to protect cellular systems from oxidative damage. These include intracellular enzymes such as superoxide dismutase, glutathione peroxidase, catalase and other peroxidases, thioredoxin reductase, the sequestration of metal ion cofactors such as Fe and Cu by binding to proteins, and endogenous antioxidants. Superoxide dismutase (SOD) enhances the otherwise slow breakdown of superoxide, forming the less toxic hydrogen peroxide, which can then interact with glutathione and ultimately form H_2O and O_2. SOD exists in a constitutively expressed form and an inducible form (MnSOD) that resides in mitochondria. MnSOD is induced by cytokines through NF-κB and may require other cofactors including nucleolar phosphosmin, an RNA-binding protein [6]. Glutathione peroxidase, the primary mitochondrial defense from hydrogen peroxide, is upregulated by p53 and hypoxia [7,8]. Catalase also degrades hydrogen peroxide, and probably has a function in cytosolic or extracellular protection from oxidants because it is absent from the mitochondria of most cells. The thioredoxin–thioredoxin reductase system is another essential component of the cellular response to oxidative stress, especially in cardiac tissue [9]. Several stressors, including inflammatory cytokines and oxidative stress, induce thioredoxin. Thioredoxin regulates protein redox status and, when activated, facilitates protein–DNA interactions. In cardiac tissue, thioredoxin expression is enhanced under conditions of cyclic hypoxia and reperfusion. Enhanced

Table 1

Equations

Oxygen radical generation

NADPH oxidase:	$2O_2 + NADPH$	$\rightarrow$	$2O_2^{\bullet-}$ (superoxide) $+ NADPH^+ + H^+$
Spontaneous conversion:	$2O_2^{\bullet-} + 2H^+$ $\rightarrow$	$[2HO_2^{\bullet}$ (hydroperoxyl radical)$] \rightarrow$	$O_2 + H_2O_2$
Superoxide dismutase:	$2O_2^{\bullet-} + 2H^+$	$\rightarrow$	$O_2 + H_2O_2$
Myeloperoxidase:	$Cl^- + H_2O_2$	$\rightarrow$	OCl^- (oxidised halide) $+ H_2O$

Reactive oxygen species secondary products

$H_2O_2 + Fe^{2+}$	$\rightarrow$	$OH^- + OH^{\bullet}$ (hydroxyl radical) $+ Fe^{3+}$
$Fe^{3+} + O_2^{\bullet-}$	$\rightarrow$	$O_2 + Fe^{2+}$
$O_2^{\bullet-} + HOCl$	$\rightarrow$	$O_2^{\bullet-} + OH^{\bullet} + Cl^-$
$H_2O_2 + OCl^-$	$\rightarrow$	$^1O_2$ (singlet oxygen) $+ H_2O + Cl^-$
$NH_3 + HOCl$	$\rightarrow$	NH_2Cl (chloramine) $+H_2O$

$$\textbf{R}\text{-CHNH}_2\text{-COOH} + HOCl \rightarrow \textbf{R}\text{-CHNHCl–COOH} +H_2O \rightarrow \textbf{R}\text{-CHO} + CO_2 + NH_4^+ + Cl^-$$
(amino acids) (chloramines) (aldehydes)

Nitrogen radical generation and secondary reactions

Nitric oxide synthetase:	arginine $+ O_2 + NADPH$	$\rightarrow$	$NO^{\bullet}$ + citrulline $+ NADP^+$
	$NO^{\bullet} + O_2^{\bullet-}$	$\rightarrow$	$ONOO^-$ (peroxynitrite)
	$ONOO^- + H+$	$\rightarrow$	$ONOOH$ (peroxynitrous acid)
	$ONOOH$ (peroxynitrous acid)	$\rightarrow$	NO_3^- (nitrate ion)
	$ONOOH$	$\rightarrow$	NO_2+ (nitronium ion)
	$ONOOH$	$\rightarrow$	$NO_2^{\bullet}$ (nitrogen dioxide radical)
	$ONOOH$	$\rightarrow$	OH^-
	$ONOOH$	$\rightarrow$	$OH^{\bullet}$ (hydroxyl radical)

Lipid peroxidation

$LH + Radical^{\bullet}$	$\rightarrow$	$L^{\bullet} + RH$
$L^{\bullet} + O_2$	$\rightarrow$	$LOO^{\bullet}$ (lipid peroxyl radical)
$LH + LOO^{\bullet}$	$\rightarrow$	$L^{\bullet} + LOOH$ (leading to lipid propagation)
$LOO^{\bullet} + TocOH$ (α-tocopherol)	$\rightarrow$	$LOOH + TocO^{\bullet}$ (chain termination)

thioredoxin expression has also been demonstrated in RA synovial fluid and tissue [10–12].

Endogenous antioxidants protect cellular systems from the damaging effects of ROS and reactive nitrogen species (RNS) reviewed in [13]. The main antioxidants are vitamin A (retinol and metabolites), vitamin C (ascorbic acid) and vitamin E (α-tocopherol). β-Carotene, a water-soluble provitamin A, is a free-radical scavenger that controls the propagation of reactive species and influences lipoxygenase activity. Vitamin C (ascorbic acid), one of the first lines of defense from oxidative stress, can prevent lipid peroxidation by trapping water-soluble peroxyl radicals before their diffusion into lipid membranes; it also

reacts with superoxide, peroxy, and hydroxyl radicals, and is important in recycling other antioxidants such as vitamin E. Vitamin E has lipid-soluble properties that allow it to act as a chain-breaking reagent in lipid peroxidation.

Evidence for oxidative stress in RA
Several lines of evidence suggest a role for oxidative stress in the pathogenesis of RA. Epidemiologic studies have shown an inverse association between dietary intake of antioxidants and RA incidence [14–17], and inverse associations between antioxidant levels and inflammation have been found [18,19]. Iron, a catalyst for hydroxyl radical production from hydrogen peroxide (see Table 1), is present in RA synovial tissue and is associated with

poorer prognosis [20]. Several groups have demonstrated increased oxidative enzyme activity along with decreased antioxidant levels in RA sera and synovial fluids [21–25]. Because of the highly reactive nature of ROS, it is difficult to directly demonstrate their presence *in vivo*. It is considerably more practical to measure the 'footprints' of ROS and RNS, such as their effects on various lipids, proteins, and nucleic acids. Thus, evidence for oxidative stress in RA has in many cases been generated by approaches that detect oxidant-induced changes to these molecules (reviewed in [1,26–28]). Studies of RA synovial fluid and tissue have demonstrated oxidative damage to hyaluronic acid [29], lipid peroxidation products [30,31], oxidized low-density-lipid proteins (LDL) [32], and increased carbonyl groups reflective of oxidation damage to proteins [32,33]. Evidence of oxidative damage to cartilage, extracellular collagen, and intracellular DNA has also been demonstrated (see below). Oxidative stress has been shown to induce T cell hyporesponsiveness in RA through effects on proteins and proteosomal degradation [34]. Finally, antioxidants and oxidative enzymes have been shown to ameliorate arthritis in animal models [35–37].

Cartilage/collagen effects

ROS and RNS damage cellular elements in cartilage directly and damage components of the extracellular matrix either directly or indirectly by upregulating mediators of matrix degradation (reviewed in [2,26]). Modification of amino acids by oxidation, nitrosylation, nitration, and chlorination can alter protein structure and impair biological function, leading to cell death. ROS impair chondrocyte responses to growth factors and migration to sites of cartilage injury; RNS, in particular NO, interfere with interactions between chondrocytes and the extracellular matrix [38]. NO can also increase chondrocyte apoptosis.

Oxygen and nitrogen radicals inhibit the synthesis of matrix components including proteoglycans by chondrocytes. In particular, NO and O_2 seem to inhibit type II collagen and proteoglycan synthesis and the sulfation of newly synthesized glycosaminoglycans. Oxygen radicals can cause low levels of collagen fragmentation and enhanced collagen fibril cross-linking. Oxygen radicals have also been shown to fragment hyaluronan and chondroitin sulfate [39,40] and damage the hyaluronan-binding region of the proteoglycan core protein, thereby interfering with proteoglycan–hyaluronan interactions [41]. In addition, ROS and RNS can damage the components of the extracellular matrix indirectly through the activation and upregulation of matrix metalloproteinases.

Oxidative damage to immunoglobulins – advanced glycation end-products

Oxidative stress occurring during inflammation can cause proteins to become non-enzymatically damaged by glyoxidation. This process, which involves primarily lysine and arginine residues, ultimately results in the generation of advanced glycation endproducts (AGE), which are stable. An example of this process is the glyoxidation of hemoglobin to hemoglobin A1c in the context of repetitive hyperglycemia. The immunoglobin molecule can also undergo similar glyoxidation to generate AGE-IgG. In the context of inflammatory arthritis, we have shown that antibodies to AGE-IgG are specifically associated with RA, whereas the actual formation of AGE-IgG is related to the intensity of the systemic inflammatory response, and is not specific to RA [42,43].

Genotoxic effects of oxidative stress

Reactive oxygen and nitrogen species directly damage DNA and impair DNA repair mechanisms. This damage can occur in the form of DNA strand breakage or individual nucleotide base damage. DNA reaction products, in particular 8-oxo-7-hydro-deoxyguanosine formed by the reaction of hydroxyl radicals (OH•) with deoxyguanosine, are elevated in leukocytes and sera of patients with RA [44,45]. This product is particularly mutagenic and cytotoxic. NO, especially in high concentrations, causes the deamination of deoxynucleotides, DNA strand breakage and oxidative damage from peroxynitrite, and DNA modification by metabolically activated *N*-nitrosamines, all of which can lead to somatic mutations.

RA tissue has evidence of microsatellite instability reflecting ongoing mutagenesis [46]. Such mutagenesis is normally corrected by DNA repair systems including the mismatch repair (MMR) system; however, the MMR system is defective in RA, probably due in part to oxidative stress. Evidence for this comes from findings of decreased expression of hMSH6, a component of the MutSα complex that is important for repair of the single base mismatches that are characteristic of oxidative stress, and increased expression of hMSH3, a component of MutSβ that is important for the repair of insertion or deletion loops. This pattern of MMR expression was reproduced by synovial fibroblasts exposed to reactive nitrate species and to a smaller extent by fibroblasts exposed to ROS, indicating a role for oxidative stress in the development of microsatellite instability in RA. The authors of this work suggest that this pattern of MMR expression might allow short-term cell survival by preventing potentially major DNA damage at the expense of minor DNA damage or that it might promote the development of a mutated phenotype having additional survival benefit.

Although somatic DNA mutations probably occur randomly through the genome, they may occur in the coding regions of functional genes. An example of this is the p53 tumor suppressor gene. The p53 tumor suppressor protein is important in containing and repairing

mutations through its effects on growth regulating genes, G1 growth arrest, interactions with DNA repair mechanisms, and apoptosis. In addition, wild-type p53 downregulates NOS and subsequent NO production through interaction with the region of the NOS2 promoter [47]. Somatic mutations of p53 have been demonstrated in RA synovium and cultured RA fibroblast-like synovio-cytes [48,49], and have been implicated in the pathogenesis of inflammatory arthritis [28]. These are primarily transitional mutations consistent with mutations resulting from oxidative deamination by nitric oxide or oxygen radicals, and are similar to those found in tumors. Importantly, there is a distinct geographical distribution of the mutations in RA synovium [50]. The distribution of p53 mutations was patchy, with most being located in the lining layer, an area distant from oxygenating vasculature and bathed in oxidant-rich synovial fluid. Specific histologic correlation was not provided; however, it is interesting to speculate that the areas with a high frequency of p53 mutations might also have lining layer hyperplasia and that these mutations contribute to the formation of the invasive pannus.

Mitochondrial DNA (mtDNA) is particularly susceptible to oxidative stress, and prolonged exposure leads to persistent mtDNA damage without effective repair, loss of mitochondrial function, cell growth arrest, and apoptosis [51]. This increased susceptibility probably relates to the proximity of mtDNA to oxidative reactive species including the lipid peroxidation products generated from inner mito-chondrial membrane lipids, which contain components of the respiratory electron transport chain, or a lack of protecting histones, or potentially inefficient repair mechanisms. The relevance of mtDNA to inflammatory arthritis is found from studies demonstrating that extracellular mtDNA is increased in RA synovial fluid and plasma [45] and that oxidatively damaged mtDNA can induce murine arthritis [52].

Lipid peroxidation
Lipid peroxidation has been implicated in the patho-genesis of cancer, atherosclerosis, degenerative diseases, and inflammatory arthritis. During lipid peroxidation, polyunsaturated fatty acids are oxidized to produce lipid peroxyl radicals that in turn lead to further oxidation of polyunsaturated fatty acid in a perpetuating chain reaction that can lead to cell membrane damage (see Table 1). Matrix degradation arising from cytokine-stimulated chondrocytes was shown to be primarily due to lipid peroxidation, and to be preventable by vitamin E, the primary antioxidant for lipids [53].

Lipid oxidation probably contributes to accelerated athero-sclerosis in RA [54–56]. Persistent local and systemic elevation of inflammatory cytokines promotes lipolysis, and the systemic release of free fatty acids contributes to the

dyslipidemia seen in RA. Oxidative stress arising from inflammatory reactions leads to the oxidation of local LDL. Oxidized LDL promotes further inflammatory changes, including local upregulation of adhesion molecules and chemokines. Advanced glycation endproducts might also contribute to this inflammation. Monocytes ingest large quantities of oxidized LDL, resulting in the formation of foam cells that are present in atherosclerotic plaques of vessels and have also been found in RA synovial fluid [57] and synovium [58].

## Role of hypoxia and reoxygenation in RA synovitis
Several lines of evidence have suggested that cycles of hypoxia/reoxygenation are important in sustaining RA synovitis. It has long been known that RA synovial fluids are hypoxic, acidotic, and exhibit low glucose and elevated lactate concentrations [59,60]. This biochemical profile is indicative of anaerobic metabolism in the synovium [61,62]. We have recently repeated the seminal experiments evaluating pO_2 levels in RA synovial fluids and found that the pO_2 levels are frequently below those detected in venous blood, with some being as low as 10 mmHg (CAH and HSE-G, unpublished work). These levels correlated with lactic acid levels. It has proven more difficult to measure pO_2 levels in RA synovium directly *in vivo*. Two studies, published in abstract form, evaluated RA synovial pO_2 with microelectrodes and found these levels to be quite low [63,64]. These data are supported by similar findings in experimental inflammatory arthritis [65]; together they support the notion that RA synovitis has the features of a chronically hypoxic microenvironment that compensates by using anaerobic metabolism.

Cellular responses to hypoxia: the role of HIF-1α
The potential role of hypoxia in RA synovitis has largely been extrapolated from studies of tumors, in which the rapidly proliferative state and high metabolic demands of the tumor cells result in areas of hypoxia generated by an imbalance between the demands and the abnormal tumor vascular supply. This hypoxic microenvironment potently stimulates tumor angiogenesis and results in phenotypic changes in the tumor cells that favor survival and growth in this environment [66,67]. The biological basis of this process has been well studied, and relates to the exquisite regulation of a key transcription factor, HIF-1α [68]. This oxygen-sensitive transcription factor orchestrates the expression of a wide spectrum of genes that serve, first, to allow the cells to use anaerobic metabolism to generate energy; second, to enhance survival and inhibit apoptosis; and third, to improve the supply of oxygen by promoting angiogenesis and increased oxygen-carrying capacity.

In view of the crucial role of HIF-1α in cellular adaptation to hypoxia, its regulation needs to be rapidly responsive to changes in the cellular oxygen supply. Although several

Figure 1
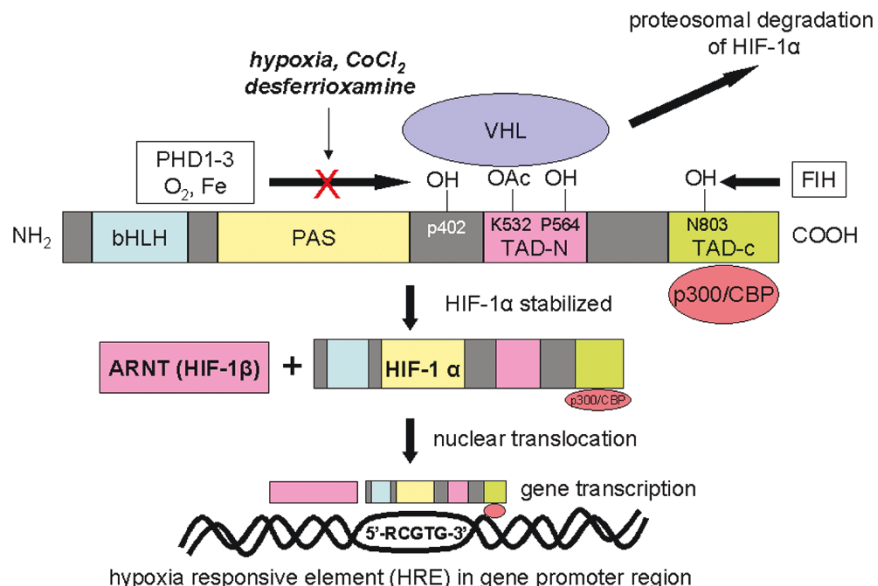
Hypoxic regulation of the hypoxia-inducible factor-1α (HIF-1α) transcription factor is primarily through inhibition of degradation. Under normoxic conditions, HIF-1α undergoes rapid proteosomal degradation once it forms a complex with von Hippel–Landau tumor suppressor factor (VHL) and E3 ligase complex. This requires the hydroxylation of critical proline residues by a family of HIF-1α-specific prolyl hydroxylases (PHD-1,2,3), which requires O_2 and several cofactors, including iron. Under hypoxic conditions, or when iron is chelated or competitively inhibited, proline hydroxylation does not occur, thus stabilizing HIF-1α and allowing it to interact with the constitutively expressed HIF-1β (aryl hydrocarbon nuclear translocator; ARNT). The HIF-1 complex then translocates to the nucleus and activates genes with hypoxia-responsive elements in their promoters. bHLH, basic helix-loop-helix; CBP, cAMP response element binding protein; FIH, factor inhibiting HIF-1α; PAS, PER-ARNT-SIM; TAD, transactivation domain.
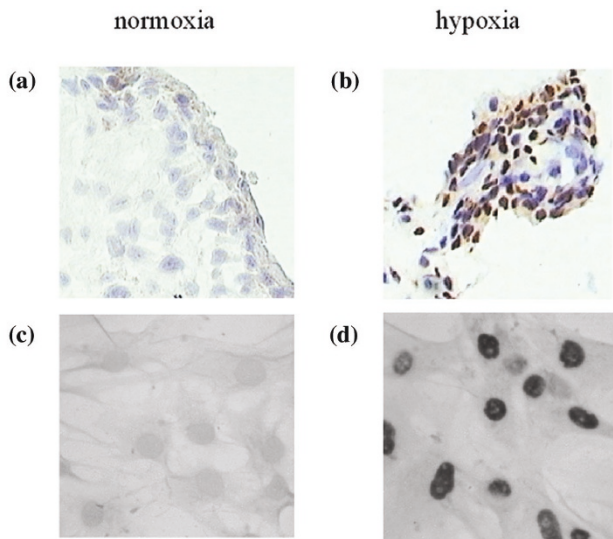
mechanisms have been proposed for oxygen sensing, it has been shown that the primary mechanism by which hypoxia directly regulates HIF-1α is by inhibiting its degradation [68]. Under aerobic conditions HIF-1α is undetectable because of a rapid process of ubiquitination and subsequent proteosomal degradation. This degradative process is mediated by von Hippel–Landau tumor suppressor factor (VHL) [69,70], which when mutated results in von Hippel–Landau syndrome, characterized by the formation of hemangiomas due to uninhibited angiogenesis. The interaction between HIF-1α and VHL requires the critical hydroxylation of two proline residues (402 and 564) and one asparagine residue (803), as well as the acetylation of a lysine residue (532) in HIF-1α [71,72]. The hydroxylation events are mediated by a family of three prolyl hydroxylases (PHD-1, PHD-2, and PHD-3) and one asparagine hydroxylase (FIH), and require O_2 and several cofactors, particularly iron and ascorbate (Fig. 1). In the absence of O_2, this critical hydroxylation becomes rate limiting, thus preventing HIF-1α from being degraded and leaving it free to bind to its constitutively expressed partner, HIF-1β (aryl hydrocarbon nuclear translocator; ARNT).

It should be noted that the degradation of HIF-1α can also be inhibited by approaches that limit the availability of iron.

Thus, cobalt chloride ($CoCl_2$), a competitive inhibitor, and desferioxamine, an iron chelator, both potently stabilize HIF-1α *in vitro* and mimic the effects of hypoxia. HIF-1α/ARNT form a complex with CBP/p300, and this complex rapidly translocates to the nucleus and transactivates genes that have a hypoxia-responsive element (HRE) in their promoters featuring the consensus motif RCGTG. Although the full complement of HRE-regulated genes are obviously present in all cells, the hypoxia-induced expression of some of these genes, such as erythro-poietin, is quite tissue specific. Other genes, such as vascular endothelial growth factor (VEGF), and genes encoding for glycolytic enzymes, are induced by hypoxic stimulation in most cells. It is interesting to speculate that glucose-6-phosphate isomerase, which as been proposed as an autoantigen in RA [73–75], is induced by hypoxia in a HIF-1α-dependent manner [76]. The list of genes that have been shown to be directly regulated by HIF-1α is shown in Fig. 2.

Thus, although there is now a well-defined group of genes that are regulated by hypoxia through HIF-1α, their patterns of expression vary in different cells and tissues. Interestingly, it has recently been demonstrated that HIF-1α is essential for the function of myeloid cells of the innate immune systems such as neutrophils and macrophages

Figure 2

Genes that have been shown to be directly regulated by hypoxia-inducible factor-1α through hypoxia-responsive elements in their promoter regions. The genes are classified on the basis on their best known functional properties. A full listing of the gene annotations is presented in the Additional file.

[77]. This study demonstrated that the regulation of glycolytic capacity by HIF-1α in these myeloid cells is crucial for the energy generation required for cell aggregation, motility, invasiveness, and bacterial killing. Of particular relevance to RA was the marked attenuation of synovitis and articular damage in an adjuvant arthritis model when HIF-1α was absent.

The effects of ROS on HIF-1α itself have been controversial [78]. One hypothesis suggests that ROS are produced by the NADPH oxidase system and serve to inhibit HIF-1α activation [79]. During hypoxia, reduced ROS formation serves to activate HIF-1α by diminished inhibition. An alternative hypothesis suggests that ROS are in fact produced by mitochondria during hypoxia and may indeed serve to stabilize HIF-1α and promote nuclear localization and gene transcription [80,81]. There is experimental evidence in support of both of these competing hypotheses, and indeed, both may be correct depending on the intensity and duration of the hypoxic stimulus, and on the cell type involved.

In addition to hypoxic regulation of HIF-1α, it has been established that cytokines and growth factors such as interleukin-1β (IL-1β), TNF-α, transforming growth factor-β (TGF-β), platelet-derived growth factor, fibroblast growth factor-2, and insulin-like growth factors are capable of stabilizing and activating this key transcription factor under normoxic conditions [82–87]. Several signaling pathways are involved, particularly the phosphoinositide 3-kinase

(PI-3K)/Akt pathway, and the mitogen-activated protein (MAP) kinase pathway. It is likely that the normoxic regulation of HIF-1α by the PI-3K/Akt pathway involves increased translation of the protein, whereas MAP kinase regulation involves phosphorylation of the molecule, which in turn increases its transactivating capacity [88,89]. The regulation of HIF-1α by NO has also recently been shown to be mediated by the MAP kinase and PI-3K/Akt pathways [89].

HIF-1α and hypoxia-regulated genes in RA synovitis
The expression of HIF-1α has been evaluated in RA and other forms of synovitis [90–92]. One study suggested that HIF-1α is widely expressed in RA synovium, and on the basis of evaluating consecutive sections it was assumed to be expressed in a cytoplasmic pattern by macrophages in both the lining and sublining areas [92]. A second study evaluated the expression of HIF-1α and the related protein HIF-2α in RA, osteoarthritis, and normal synovium, and found them to be widely expressed in both RA and osteoarthritis but not in normal synovium [90]. The synovial expression of HIF-1α in this study was in a mixed nuclear and cytoplasmic pattern, and was seen in most lining cells, stromal cells, mononuclear cells, and blood vessels. On the basis of these findings, the authors suggested a role for hypoxia and HIF-1α in the pathogenesis of both RA and osteoarthritis.

Our own studies of synovial HIF-1α expression have suggested a more limited, patchy pattern of nuclear

Figure 3

Expression of hypoxia-inducible factor-1α (HIF-1α) in RA synovium and fibroblast-like synoviocytes under normoxic and hypoxic conditions. **(a)** Under normoxic conditions, HIF-1α expression in fresh synovial explants was patchy and confined to some cells in the lining layer. **(b)** When fresh RA tissue explants were cultured in hypoxic conditions (1% O$_2$), nuclear staining for HIF-1α was readily detected in the lining cells. **(c, d)** A similar pattern of expression was seen in fibroblast-like synoviocytes where under normoxic conditions no HIF-1α staining was detected (c), whereas under hypoxic conditions intense nuclear staining was seen maximally at 4–6 hours (d). Reproduced, with permission, from [91].

expression that was confined primarily to the lining cells of RA tissues with a particularly hyperplastic lining layer [91] (Fig. 3). Indeed, when we exposed fresh synovial tissue explants to hypoxic culture conditions, the nuclear expression of HIF-1α increased markedly in the lining cell layer, in a manner analogous to that seen in cultured synovial fibroblasts. It should be noted that our immunohistology studies were performed on snap-frozen sections of synovium with the use of three commercially available anti-HIF-1α antibodies. In contrast, the two other studies used archival synovial tissue that had been deparaffinized and then subjected to antigen retrieval techniques. It is currently not clear whether these technical considerations are sufficient to explain these discrepant findings.

The presence of regional HIF-1α expression in hyperplastic areas of the RA lining layer would be consistent with a dynamic process in which the lining cells in these areas, being the furthest removed from a precarious and insufficient vascular supply in the sublining areas, are subjected to fluctuating oxygen levels, resulting in repetitive cycles of hypoxia and reperfusion. Moreover, such a regional distribution of HIF-1α expression would also be in keeping with the known rapid stabilization and nuclear translocation of HIF-1α under transient hypoxic conditions, which is followed by equally rapid degradation of this transcription factor when relative normoxia is re-established [93].

The expression of several HIF-1α-regulated genes has been explored in RA synovitis, in particular angiogenesis mediators such as VEGF and the angiopoietins. VEGF has been shown to be upregulated in the serum, synovial fluid, and synovium of patients with RA [94–98]. Moreover, clinical response to TNF-α inhibitors is associated with a decrease in systemic and synovial VEGF levels, this being attributed to inhibition of synovial angiogenesis [96,99]. At the cellular level, the regulation of VEGF expression is complex. We and others have shown that cytokines abundant in RA synovium, such as TNF-α, IL-1β, and TGF-β, interact with hypoxia in an additive manner to induce VEGF expression by fibroblast-like synoviocytes [91,100]. The interaction at the level of the VEGF promoter between HIF-1α and SMAD3, the latter being the mediator of TGF-β transcriptional regulation, has been demonstrated [101]. Similarly, the angiopoietins Ang1 and Ang2, and their cellular receptor Tie2, which are all widely expressed in RA synovitis, are regulated by both hypoxia and TNF-α [102–105]. These observations underscore the complexity of transcriptional regulation in a chronic inflammatory microenvironment such as RA synovium, and indicate that the regulation of specific genes by hypoxia occurs in the context of multiple other regulatory pathways, particularly the NF-κB pathway.

Hypoxia, or hypoxia and reoxygenation?
Studies of RA synovium *in vivo* have suggested that synovial perfusion is influenced directly by high intraarticular pressures that are further increased by movement [106–108]. On the basis of these observations, it can therefore be proposed that intermittent joint loading with ambulation, especially in the setting of an effused joint, enhances local joint hypoxia, which in turn is followed by reoxygenation when the joint is unloaded. A predicted consequence of such cycles of hypoxia and reoxygenation would be cycles of HIF-1α expression and the genes it regulates, followed by repetitive bursts of ROS formation. The ROS generated serve as a stimulus for NF-κB activation, probably through effects on upstream kinases [109,110]. This includes effects on the dissociation of NF-κB from its inhibitor IκB (which requires oxidation), the regulation of IκB degradation, and the binding of NF-κB to DNA (which requires a reducing environment). Activation of NF-κB serves to induce the expression of multiple proinflammatory genes, many of which are also regulated by HIF-1α [78,111]. This interaction is summarized in Fig. 4. The resultant changes in gene and protein expression are complex and vary in different cell types, but overall can be expected to promote inflammation, angiogenesis, and enhanced cell survival, all cardinal features of RA synovitis.

Figure 4
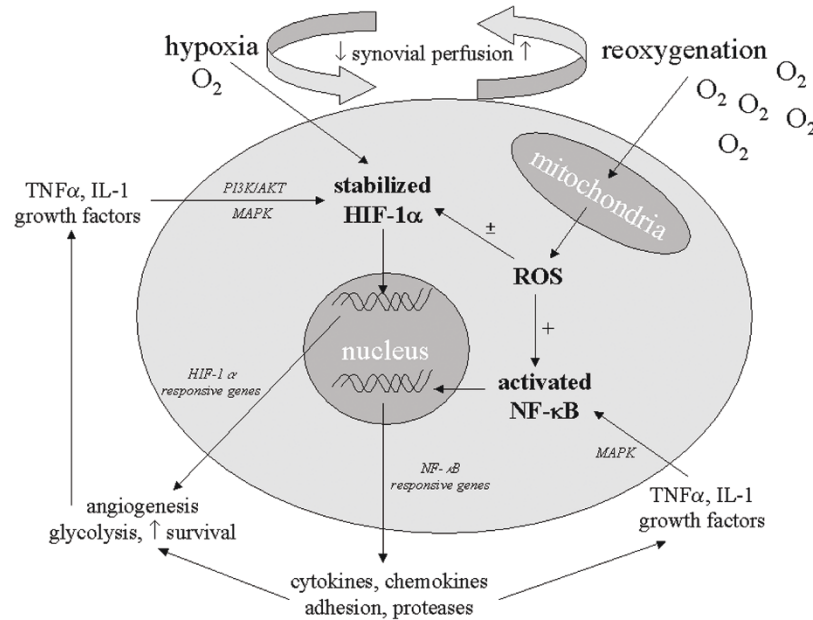
Regulation of the hypoxia-inducible factor-1α (HIF-1α) and nuclear factor-κB (NF-κB) pathways by reactive oxygen species (ROS) and cytokine stimulation. The complex and interrelated activation of these two critical transcription factors is central to most of the processes that sustain synovitis in rheumatoid arthritis, such as endothelial activation, leukocyte recruitment, angiogenesis, and enhanced cell survival. IL, interleukin; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; TNF, tumor necrosis factor.

The sequelae of hypoxia and reoxygenation have been addressed in vascular models, and some limited experimental evidence has addressed this question in RA synovium [112]. Interestingly, the vascular models of hypoxia and reoxygenation have demonstrated a phenomenon that has been termed preconditioning. This describes a process whereby a cell or a tissue becomes resistant to subsequent hypoxic episodes after transient exposure to a hypoxic episode. The biological basis of preconditioning continues to be defined, and might involve signaling by Akt [113] and/or extracellular signal-related kinase 1/2 [114], and possibly an upregulation of PHD-2 during the hypoxic phase [115]. It is currently not known whether some form of preconditioning occurs in RA synovitis, and whether this promotes the survival of cells in this oxidatively stressed microenvironment.

Therapeutic considerations
Targeting ROS with antioxidants
Various forms of antioxidant therapy have demonstrated promising results in experimental arthritis models [35–37]. The polyphenolic fraction of green tea containing potent antioxidants prevents collagen-induced arthritis [116]. The beneficial effects seem to be due to the catechin epigallocatechin-3-gallate (EGCG), which inhibits IL-1β-mediated inflammatory effects, including NOS and NO production by human chondrocytes [117], and inhibits MMP activity [118,119].

There is widespread availability and interest in the use of antioxidant supplementation by patients with inflammatory arthritis, although proof of efficacy is modest. A traditional Mediterranean diet relatively high in antioxidants improved RA disease activity and functional status after 3 months compared with a standard 'Western' diet, although clinical improvement was not associated with any significant change in plasma levels of antioxidants [16,120]. In a separate study of patients with RA, supplementation with antioxidants vitamin A, E, and C increased plasma antioxidant levels with a corresponding decrease in malondialdehyde, a marker of oxidative stress; however, a clinical response was not reported [121]. Specific supplementation of oral vitamin E, the major lipid-soluble antioxidant in human plasma, erythrocytes, and tissue, had no effect on RA disease activity or indices of inflammation but did improve pain, suggesting a role in central analgesia mechanisms [122].

Targeting angiogenesis
It has been proposed that the formation of destructive RA pannus is dependent on synovial angiogenesis, in a manner analogous to locally invasive tumors. As is the case with many tumors, hypoxia has a central role in regulating this angiogenic process. On this basis, inhibition of synovial angiogenesis has been proposed as a rational therapeutic strategy, and several angiogenesis inhibitors have been shown to have favorable effects in

animal models (reviewed in [123]). As mentioned earlier, it has been suggested that the therapeutic responses to TNF-α inhibition might be attributable, at least in part, to an inhibition of angiogenesis [99].

An alternative hypothesis suggests that, rather than representing a tumor-like proliferative process that outgrows its vascular supply, RA pannus represents a non-healing synovial wound that is prevented from resolution by an inadequate vascular supply. Hypoxia has long been proposed as an important stimulus in wound healing [124]. Moreover, hypoxia and HIF-1α serve to stimulate genes that are involved in wound repair and the formation of granulation tissue, a process critically dependent on angiogenesis [125–129]. Interestingly, the expression of HIF-1α protein does not occur during the initial inflammatory process but becomes evident within 1–5 days of wounding, and seems to have a prominent role in the subsequent tissue healing. If RA synovitis does have many of the features of a non-healing wound, inhibition of angiogenesis would conceptually not represent an appropriate strategy and indeed might have deleterious effects, depending on the stage of the synovitis being treated.

Targeting HIF-1α and hypoxic cells
Our understanding of cellular and tissue responses to changes in oxygen tension has increased markedly over the past decade. The central role of HIF-1α in mediating hypoxic responses has suggested new therapeutic opportunities, particularly in cancer and cardiovascular medicine [130,131]. Small molecules targeting the HIF-1α pathway are currently being developed and show considerable promise in cancer models. It should be noted that many cancer cells overexpress HIF-1α on a genetic basis, a phenomenon that presumably enhances their survival in hypoxic environments [131]. It is not clear whether an analogous situation exists in RA pannus. As mentioned above, studies evaluating the expression of HIF-1α in RA synovitis have not provided a consistent picture, although all studies so far have pointed to the synovial lining layer as the main site of HIF-1α expression. It is not clear whether this expression is 'physiological', in response to poor tissue oxygenation, or pathological, as seen in many tumors. Moreover, chondrocytes that function in a physiologically hypoxic environment are critically dependent on HIF-1α for normal development and maintenance of cartilage integrity [132–136]. Thus, targeting HIF-1α in an articular disorder such as RA remains a conceptually challenging proposition requiring considerably more experimental data.

An alternative approach is to target hypoxic cells by using their 'reducing' intracellular microenvironment to generate toxic metabolites locally from specific drugs [137]. These 'bioreductive' drugs would thus be more toxic to hypoxic

than normoxic cells. Alternatively, such drugs could serve as carriers for delivering anti-inflammatory compounds to target tissues. One such bioreductive drug, metronidazole, has been proposed as potentially being useful for this purpose, although a controlled clinical trial had produced mostly disappointing results [138].

Conclusions
Repetitive cycles of hypoxia and reoxygenation, along with oxidants produced by phagocytic cells such as macrophages and neutrophils, lead to chronic oxidative stress in the RA synovial microenvironment. The ROS that are generated damage proteins, nucleic acids, lipids, and matrix components, and serve to amplify signaling pathways that sustain the synovitis. HIF-1α and NF-κB are key transcription factors that respond to changes in cellular oxygenation and that orchestrate the expression of a spectrum of genes that are critical to the persistence of the synovitis. An understanding of the complex interactions involved in these pathways may allow the development of novel therapeutic strategies for RA.

Additional file

The following Additional file is available online:

Additional file 1

An Excel file containing a table that gives details of the gene annotations used in Fig. 2.
See http://arthritis-research.com/content/supplementary/ar1447-s1.xls

Competing interests
The author(s) declare that they have no competing interests.

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