Role of reactive oxygen species in rheumatoid arthritis synovial T lymphocytes

Remans, Philip Herman Jozef

Chapter1:
General Introduction

1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory joint disease affecting about 1% of the population. Although new diagnostic tools (such as detection of anti-CCP antibodies) and novel therapies (such as anti-TNFα therapy) have contributed to a greater understanding of the pathogenesis of RA, its etiology remains unknown. The inflamed synovium in patients with RA is characterised by infiltration with a diverse spectrum of leucocytes: predominantly macrophages and T lymphocytes (comprising 30-40% of the cellular infiltrate each), but also B cells and dendritic cells, each cell type playing a specific role in the chronic inflammation. In the course of the inflammation, proliferation of synovial fibroblasts is induced together with the production of proteolytic enzymes, which leads to the destruction of adjacent cartilage and bone. If left untreated, these processes lead to deformity and disability.
Recent clinical studies with abatacept® (CTLA4Ig), a selective inhibitor of T cell costimulation have demonstrated that T cells play a central role in RA (1). T lympocytes isolated from the rheumatic joint display a number of specific characteristics. While expressing markers of recent activation, including CD45RO and CD69 and proinflammatory surface receptors such as CD154 and TNF-α receptor (2,3), T cells obtained from SF exhibit low proliferative and low cytokine-responses after T cell receptor (TCR)-triggering (4). In recent years, several important aspects of the molecular basis underlying SF T cell hyporesponsiveness have been elucidated. It was recently reported that the hyporesponsive state of SF T cells was related to chronic oxidative stress in these cells (4,5). T cell hyporesponsiveness was associated with decreased intracellular levels of glutathione (GSH) (5), an intracellular nonprotein thiol with both oxidant scavenging and redox regulating capacities. In vitro replenishment with the antioxidant *N*-acetyl-L-cysteine (NAC) restored GSH equilibrium concomitant with partial rescue of SF T lymphocyte hyporesponsiveness and restoration of TCR-dependent signaling defects. In contrast, treatment of T cells from healthy volunteers with DL-buthionine (*S,R*)-sulfoximine (BSO) results in depletion of intracellular GSH and mimicked the observed hyporesponsiveness and most of the signaling defects (5,6).

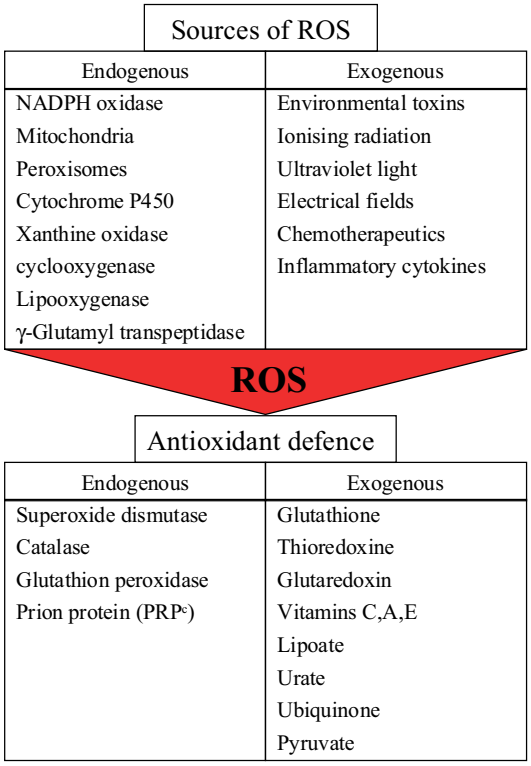
2. Oxidative stress

2.1 Introduction

Molecular oxygen (dioxygen; O_2) is essential for the survival of all aerobic organisms. In mitochondria, energy is generated by the consumption of oxygen and the reduction of oxygen to water by the transfer of four electrons without formation of intermediates. In this process of oxidative phosphorylation the oxidoreduction energy of mitochondrial electron transport is converted to the high-energy phosphate bond of ATP via a multicomponent NADH dehydrogenase enzymatic complex. Partially reduced and highly reactive metabolites of O_2 are formed during these (and other) electron transfer reactions. These O_2 metabolites include superoxide anion ($O_2\cdot$) and hydrogen peroxide (H_2O_2), formed by one- and two-electron reductions of O_2, respectively. In the presence of transition metal ions, the even more reactive hydroxyl radical ($OH\cdot$) can be formed. These partially reduced metabolites of O_2 are often

referred to as "reactive oxygen species" (ROS) due to their higher reactivities relative to molecular O_2.

ROS from mitochondria and other cellular sources have been traditionally regarded as toxic by-products of the cellular metabolism with the potential to cause damage to lipids, proteins, and DNA (7). To protect the host against the damaging effects of ROS, many antioxidative defense mechanisms have evolved. In addition to several antioxidant enzymes such as superoxide dismutase (which reduces $O_2\cdot$ to H_2O_2), catalase, and glutathione peroxidase (which reduces H_2O_2 to H_2O), a broad range of non-enzymatic scavengers exists. “Oxidative stress” may be broadly defined as an imbalance between oxidant production and the antioxidant capacity of the cell to prevent oxidative injury, and is thought to contribute to the pathogenesis of a number of human diseases including RA, atherosclerosis, pulmonary fibrosis, cancer, neurodegenerative diseases, and ageing.



During the past decade however, reduction-oxidation (redox) reactions that generate ROS (including H_2O_2, $O_2\cdot$ and $OH\cdot$) have been identified as important chemical mediators in the regulation of signal transduction processes involved in cell growth and differentiation (reviewed in 8-17). A wide range of ligand-receptor interactions has been shown to generate intracellular ROS. ROS can activate a variety of members of signalling pathways such as protein kinases, protein phosphatases and transcription factors. Although ROS are generated intracellularly by several sources, including mitochondria, the NADPH oxidases in particular have been related to receptor-mediated signalling. The rapid kinetics of activation and inactivation and the intrinsic characteristics of free radicals make them ideal intracellular messengers, that allow a tight up- and downregulation of intracellular ROS levels within the short time required for the transduction of signals from the plasma membrane to the nucleus.

From one point of view the delicate intracellular balance between oxidising and reducing equivalents reveals a complex interplay regulating cell proliferation versus cell cycle arrest or apoptosis. From another point of view it offers a potential interesting target for therapeutic intervention to module cellular function.

2.2 Chemistry of ROS.

The chemical structure of ROS influences the ability of different species to react with specific cellular substrates within the microenvironment in which they are produced. ROS-substrate reactions are likely to form the basis for our understanding of ROS specificity and their mechanisms of action. ROS are often referred to as free radicals, but this is incorrect because not all ROS are free radicals. A free radical is defined as a chemical species possessing one or more unpaired electrons in one of its molecular orbitals. Due to the unpaired electron, free radicals are very unstable and will react with any atom or molecule in their vicinity.

Free radicals can be formed via 3 mechanisms: (a) by the homolytic cleavage of a covalent bound, with each fragment retaining one of the pair of electrons; (b) by the loss of a single electron (oxidation); (c) by the addition of a single electron (reduction).

In biological systems, the most important free radicals are derivatives of oxygen.

Single-electron reduction of oxygen either by enzymatic catalysis or by "electron leaks" from various electron transfer reactions will produce superoxide:

(1) $O_2 + e^- \rightarrow O_2^{-\bullet}$

In contrast to its remarkable stability in many organic solvents, $O_2\cdot$ in aqueous solution is short-lived. This "instability" in aqueous solutions is based on the rapid dismutation of superoxide to hydrogen peroxide:

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

In mammalian cells this reaction is additionally catalysed by the enzyme superoxide dismutase (SOD). SOD speeds up this reaction almost 10^4-fold. This implies that any reaction of $O_2\cdot$ in aqueous solution will be in competition with SOD or, in its absence, with the spontaneous dismutation reaction itself. Thus in most biological systems, generation of $O_2\cdot$ usually results in the formation of H_2O_2.

Although in eukaryotic cells dismutation of $O_2\cdot$ probably accounts for much of the H_2O_2 production, H_2O_2 can also be formed by direct two-electron reduction of O_2, a reaction mechanism shared by a number of flavoprotein oxidases:

(3) $O_2 + 2e^- + 2H^+ \rightarrow H_2O_2$

Hydrogenperoxide itself is not a free radical, but because of its involvement in the production of free radicals, it falls in the category of reactive oxygen species (ROS). In the presence of transition metals (i.e. metal ions with variable oxidation numbers, for example iron as Fe^{2+}/ Fe^{3+}, or copper as Cu^+/ Cu^{2+}) hydrogen peroxide can easily break down to produce the most reactive and damaging of the oxygen free radicals, the hydroxyl radical ($^{\bullet}OH$): this equation is often referred to as the Fenton reaction.

(4) $H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$.

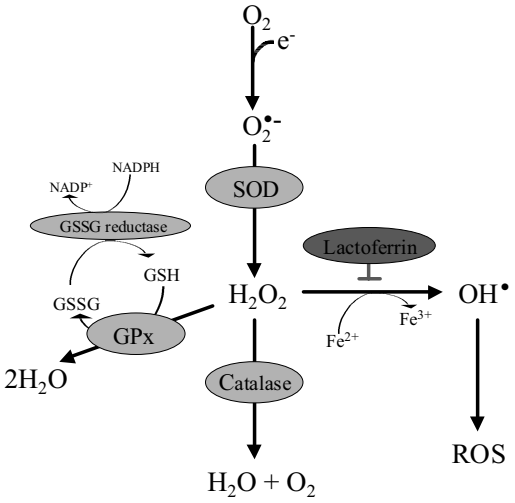
Although H_2O_2 is relatively stable and can diffuse through plasma membranes, hydrogen peroxide generally is rapidly converted into H_2O by catalase (mainly localised in peroxisomes) or glutathione peroxidase (GSH-Px; cytosolic localisation).

(5) $2H_2O_2 \rightarrow$ catalase $\rightarrow 2H_2O + O_2$

Catalase is one of the most efficient enzymes known. It is so efficient that it cannot be saturated by H2O2 at any concentration. The selenium-containing Glutathione peroxidases are also reactive towards fatty acids hydroperoxides and is dependent on the glutathione (GSH) recycling system, in which the oxidised glutathione (GSSG) is rapidly reduced back to the tripeptide GSH (γ-glutamyl-cysteinyl- glycine) by action of the enzyme GSH reductase, and through consumption of NADPH. Glutathione as such can be considered the central antioxidant in

regulating the intracellular redox state, through its critical role in the removal of peroxides and other reactive metabolic intermediates, either by directly inactivating them or by acting as a substrate for the enzymes which catalyse their removal.

Figuur 1: Schematic overview of ROS metabolism.
SOD: superoxide dismutase.
GPx: GSH peroxidase



2.3 The production of free radicals in cells.

The concept that ROS act as regulators of cell function, e.g. by acting as signalling molecules, has gained significant recognition over the past several years from studies done in laboratories worldwide. The evidence supporting this concept is based largely on the following criteria: *1*) growth factors and cytokines are capable of generating ROS in a number of different cell types, *2*) antioxidants and inhibitors of ROS-generating enzymatic systems block specific growth factor- and/or cytokine-activated signalling events or physiological effects, and *3*) exogenous addition of oxidants activates the same cytokine- and/or growth factor-mediated signalling pathway or produces the same physiological effects.

2.3.1 Ligand induced ROS production

In most cells a variety of cytokines, growth factors and hormones that bind receptors of different classes have been reported to generate intracellular ROS, following ligation to their respective receptor (Table 1).

Table 1

Ligand	Reactive Species	Enzymatic source	Cell or tissue	Functional (Or pathologic?) effects	Ref. No.
Cytokines					
TNF-α	unspecified	Mitochondria	Fibrosarcoma	NF-κB activation, IL6 induction Cytotoxicity	117
	$O_2^{\bullet}$, H_2O_2	NAD(P)H oxidase	Fibroblasts	unknown	118
	$O_2^{\bullet}$	unknown	Endothelial cells	VCAM, ICAM expression	119-121
	$O_2^{\bullet}$	NAD(P)H oxidase	Mesangial cells	MCP-1, CSF-1 expression	122
	H_2O_2	Flavoprotein oxidase	Chondrocytes	Mitogenesis	123
IL-1	$O_2^{\bullet}$, H_2O_2	NAD(P)H oxidase	Fibroblasts	unknown	124
	$O_2^{\bullet}$	NAD(P)H oxidase	Endothelial cells	MCP-1, IL-6	119
IFN-γ	$O_2^{\bullet}$	unknown	Endothelial cells	MCP-1, IL-6, VCAM	125, 126
Receptors tyrosine kinases					
PDGF	H_2O_2	unknown	BALB/3T3 cells	"competence factor" for cell growth	127
	H_2O_2	Flavoprotein oxidase	Smooth muscle cells	Mitogenesis, MAPK activation	128
	$O_2^{\bullet}$	unknown	NIH/3T3 cells	NOS expression, PGE_2 release	129
	$O_2^{\bullet}$	Flavoenzyme	Smooth muscle cells	NF-κB-dependent MCP-1 induction	130
EGF	H_2O_2	unknown	Epidermoid carcinoma	Tyrosine phosphorylation, cell growth	131
	Unspecified	unknown	HaCa T cell line	JNK activation	132
	H_2O_2	unknown	Hepatocytes	Carcinogenesis, apoptosis	133, 134
	H_2O_2	lipoxygenase	Human keratinocytes	PLA_2, MAPK activation	135
	$O_2^{\bullet}$	lipoxygenase	PC12	Cell growth	136
FGF-2	H_2O_2	Flavoprotein oxidase	Chondrocytes	Induction of c-fos	137
	$O_2^{\bullet}$	unknown	Lung fibroblasts	Mitogenesis	138
Receptor serine/threonine kinases					
TGF-β1	H_2O_2	unknown	Mouse osteoblasts	Growth inhibition; Erg1-induction	139
	H_2O_2	unknown	Endothelial cells	MAC-1	140
	H_2O_2	NADH oxidase	Lung fibroblasts	Apoptosis	141
	H_2O_2	unknown	Hepatocytes	Apoptosis	142
	H_2O_2	Suppression of AOE	Pancreatic beta cells	Apoptosis	143
G-coupled receptors					
Angiotensin II	$O_2^{\bullet}$, H_2O_2	NAD(P)H oxidase, $p22^{phox}$	Smooth muscle cells	Cell hypertrophy, p38 activation	144
	$NO^{\bullet}$	Nox4	Mesangial cells	PK B	145
	H_2O_2	PLD dependent oxidase	Smooth muscle cells	Proliferation, hypertrophy	146
	$O_2^{\bullet}$	NAD(P)H oxidase	Endothelial cells	NO induction	147

MCP-1, monocyte chemoattractant protein-1; CSF-1, colony-stimulating factor-1 HSP27, 27-kDa heat shock protein; COX-2, cyclooxygenase-2 PDGF, platelet-derived growth factor; MAPK, mitogen-activated protein kinase; NOS, nitric oxide synthase; EGF, epidermal growth factor; JNK, c-Jun NH_2-terminal kinase; PLA_2, phospholipase A_2; HB-EGF, heparin-bound EGF; FGF-2, fibroblast growth factor-2; IGF-I, insulin growth factor I; HGF, hepatocyte growth factor; NO, nitric oxide; TGF, transforming growth factor; PLD, phospholipase D; PK B, proteine kinase B

2.3.2 Cellular sources of ROS

(1) Mitochondria

As stated earlier, any electron-transferring protein or enzymatic system can result in the formation of ROS as "by-products" of electron transfer reactions. This "unintended" generation of ROS in mitochondria accounts for ~1-2% of total O_2 consumption under reducing conditions. Due to high concentrations of mitochondrial SOD, the intramitochondrial concentrations of $O_2\cdot$ are maintained at very low steady-state levels. Thus unlike H_2O_2, which is capable of diffusing across the mitochondrial membrane into the cytoplasm, mitochondria-generated $O_2\cdot$ is unlikely to escape into the cytoplasm. However, the potential for mitochondrial ROS to mediate cell signalling has gained significant attention in recent years, particularly with regard to the regulation of apoptosis (18-22). There is evidence to suggest that tumor necrosis factor (TNF-α) and interleukin (IL)-1-induced apoptosis may involve mitochondria-derived ROS (23-25). It has

also been suggested that the mitochondria may function as an "O_2 sensor" to mediate hypoxia-induced gene transcription (26,27).

(2) Endoplasmatic reticulum

Another site of electron transport is the endoplasmatic reticulum (ER) where electrons leak from the NADPH P450 reductase. Smooth ER contains enzymes that catalyse a series of reactions to detoxify lipid-soluble drugs and other harmful metabolic products. The enzyme can oxidise unsaturated fatty acids and xenobiotics and reduce molecular O_2 to produce $O_2\cdot$ and/or H_2O_2 (28). Although there does not appear to be a direct link between ER-derived oxidants and growth factor signalling, there is evidence for redox regulation of ER-related functions such as protein folding and secretion (29,30).

Nuclear membranes also contain cytochrome oxidases and electron transport systems that resemble those of the ER but the function of which is unknown. It has been postulated that electron "leaks" from these enzymatic systems may give rise to ROS that can damage cellular DNA in vivo (31).

(3) Soluble enzymes

In addition to intracellular membrane-associated oxidases, soluble enzymes such as hypoxanthine/xanthine oxidase oxidase, aldehyde oxidase, flavoprotein dehydrogenase and tryptophan dioxygenase can generate ROS during catalytic cycling. The most extensively studied of these is the $O_2\cdot$-generating xanthine oxidase, which can be formed from xanthine dehydrogenase after tissue exposure to hypoxia (32). Xanthine oxidase is widely used to generate $O_2\cdot$ in vitro to study the effect of ROS on diverse cellular processes; however, no studies have implicated a direct physiological role for endogenous xanthine oxidase in cell signalling.

(4) Lipid metabolism

In contrast to these relatively recent reports of pyridine- and flavin-linked oxidase, enzymes involved in phospholipid metabolism have been known to exist for several decades. Membrane phospholipids, in addition to their structural role in providing membrane integrity, are substrates for the action of the phospholipases (PLs) PLA_2, PLC, and PLD. Although these enzymes are important for the generation of lipid second messengers, they generally have not been associated with ROS production in nonphagocytic cells.

PLA_2 hydrolyses phospholipids to generate arachidonic acid. Arachidonic acid then forms the substrate for cyclooxygenase- and lipoxygenase (LOX)-dependent synthesis of the four major classes of eicosanoids: prostaglandins, prostacyclins, thromboxanes, and leukotrienes. These synthetic pathways encompass a series of oxidation steps that involve a number of free radical intermediates. Arachidonic acid metabolism, including the LOX pathway, which leads to leukotriene synthesis, has been reported to generate ROS (33). Recently, both phospholipase and LOX activity have also been implicated in redox-regulated signalling by ANG II (34,35), epidermal growth factor (EGF) (36), and IL-1 (37). Also, TNF-α induced apoptosis has been reported to be mediated by a LOX-dependent but mitochondrial ROS-independent mechanism (38).

(5) NADPH oxidase

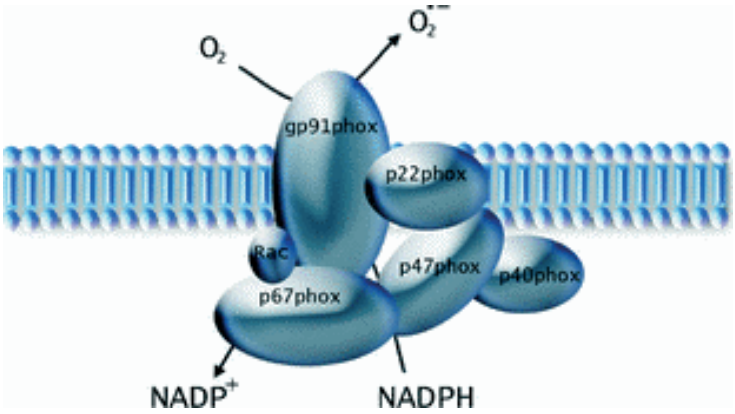
By far the most important multienzyme complex involved in the generation of ROS in signal transduction pathways is the NADPH oxidase. This plasma membrane-associated oxidase has been implicated as the source of most growth factor- and/or cytokine-stimulated oxidant production (39-44), although the precise enzymatic sources have yet to be fully characterised.

Oxidative burst in phagocytic cells

In neutrophils the membrane bound NADPH oxidase produces toxic ROS, which are used in the destruction of microbial pathogens. The transfer of electrons occurs from NADPH on the inner aspect of the plasma membrane to O_2 on the outside. During phagocytosis, the plasma membrane is internalised as the wall of the phagocytic vesicle, with what was once the outer membrane surface now facing the interior of the vesicle. This targets the delivery of $O_2\cdot$ and its reactive

metabolites internally for localised microbicidal activity (45). The neutrophil enzyme consists of 4 major subunits: a plasma membrane spanning cytochrome b-558 composed of the subunits gp91-phox and p22-phox and the 2 cytosolic components p47-phox and p67-phox. Upon stimulation, the small G protein Rac2 (Rac1 in mouse macrophages) mediates the assembly of the enzyme complex where the cytosolic components must join with the membrane bound heterodimer cytochrome b-558 for activation of the NADPH oxidase.

Figure 2: NADPH oxidase complex



The NADPH oxidase of non-phagocytic cells

A ligand-activated enzyme with NADPH/NADH oxidase activity has been shown to be present in a variety of non-phagocytic cells including endothelial cells (46,47), smooth muscle cells (48-50), chondrocytes (51, 52) and fibroblasts (53). However, whether all components of the NADPH oxidase of the phagocyte are expressed is still not clear.

In vascular smooth muscle cells angiotensin II (ANG II)-induced hypertension is mediated, at least in part, by direct interactions between $O_2\cdot$, generated by a NAD(P)H-like oxidase, and $p22^{phox}$ has been shown to be a functional component of this ANG II-stimulated oxidase (54). TNF-α also stimulates $O_2\cdot$ production in vascular smooth muscle cells by a $p22^{phox}$-based NADH oxidase and appears to upregulate $p22^{phox}$ gene expression in these cells (55). However, the smooth muscle cells appear to express $p22^{phox}$, $p47^{phox}$ but not $gp91^{phox}$ (56). More recently, Suh et al. (57) demonstrated that *Nox-1* (formerly mox-1for mitogenic oxidase), a gene encoding a homologue of the catalytic subunit of the phagocytic $gp91^{phox}$, is expressed in a number of tissues including vascular smooth muscle. In this study, *Mox-1* expression in NIH/3T3 cells was associated with increased $O_2\cdot$ production, serum-stimulated cell growth, and a transformed phenotype. This study suggests that an $O_2\cdot$-generating oxidase similar (but not identical) to the phagocytic NADPH oxidase is present in some nonphagocytic cells where it functions primarily as a regulator of cellular growth responses.

2.3.3 Signalling pathways leading to ROS production in non phagocytic cells

Although a number of growth factors and cytokines binding to different classes of receptors have been shown to raise intracellular ROS, little is known about intracellular pathways leading to ROS generation.
ROS generation in response to cytokine receptor stimulation has been reported for TNF-α, IL-1, and interferon-γ and has been mainly related to the induction of apoptotic pathways resulting in cell death. There is no consensus on the specific species produced, the enzymatic source, or the site of generation of ROS for these cytokines. In the case of TNF, recent reports (58-62) suggest that a mitochondrial source of ROS is required for activation of the transcription factor nuclear factor (NF)-B and NF-B-dependent gene transcription.
In contrast to growth factors, which elicit mitogenic stimuli by the generation of ROS through NADPH-oxidase-like enzymes, it is suggested that TNF-α generates ROS through the mitochondrial respiratory chain during activation of mitochondrial apoptotic pathways (23-25).
ROS production via an NADPH oxidase generally is associated with mitogenesis and differentiation. As mentioned, activation of the oxidase, however, requires the additional participation of Rac2 for assembly of the multi-complex enzyme. During activation, Rac2 binds GTP and migrates to the plasma membrane along with the cytosolic components to form the active oxidase complex. A requirement for Rac1 in the activation of the mitogenic oxidase has been demonstrated in nonphagocytic cells (63,64). The insert region in Rac1 (residues 124-135) appears to be essential for $O_2\cdot$ production and stimulation of mitogenesis in quiescent fibroblasts but not for Rac1-induced cytoskeletal changes or activation of Jun kinase (64).
Another GTP-binding protein, p21Ras, appears to function upstream from Rac1 in oxidant-dependent mitogenic signalling (65,66). Stable transfection of the same Ras plasmid ($H\text{-}Ras^{V12}$) in fibroblasts induced cellular transformation and constitutive production of large amounts of $O_2\cdot$ (65). Sundaresan et al. (66) showed that expression of dominant negative Rac1 not only inhibited the growth factor- and/or cytokine-generated rise in intracellular ROS in NIH/3T3 cells but also ROS production in cells that overexpress a constitutively active isoform of Ras ($H\text{-}Ras^{V12}$).

2.3.4 Molecular Targets of ROS

Although ROS have been demonstrated to be involved in many signal transduction pathways the molecular targets are not yet precisely defined. There is growing evidence, however, that redox regulation might occur at multiple levels in the signalling pathways from receptor to nucleus.

Protein Tyrosine Kinases and Phosphatases.

Although binding of growth factors to their receptors and the subsequent activation of their receptor tyrosine kinase (RTK) are at the top of signalling pathways and are generally upstream from intracellular generated ROS, receptor kinases and phosphatases themselves may also be targets of oxidative stress. Oxidants such as H_2O_2, vanadate and their reaction product pervanadate have been shown to induce phosphorylation of the epidermal growth factor (EGF) receptor and the platelet derived growth facto (PDGF) (67-69). Also, lysophatidic acid-induced activation of the EGF receptor appears to be mediated by the intermediate formation of ROS (70). A study by Knebel et al. (71) suggests that the mechanism of these effects may be related to ROS-mediated inhibition of the dephosphorylation of RTKs by inactivation of membrane-bound protein tyrosine phosphatase(s).
Not only RTK's, but also many other PTK such as non-RTKs belonging to the Src family (Src kinases) are activated by free radicals. H_2O_2, pervanadate and UV light have been shown to induce tyrosine phosphorylation and the activation of many PTK's including Lck (72,73), Fyn (74) and ζ-associated polypeptide of 70kDa (ZAP-70)(75) in T cells. Although oxidative stress apparently stimulates many PTK's in cells, there is no evidence so far that PTK's are direct targets of H_2O_2. The study by Knebel et al. (71) suggests that the mechanism of these effects may be related to ROS-mediated inhibition of the dephosphorylation of RTKs by inactivation of membrane-bound protein tyrosine phosphatase(s)'s PTP's.

The reports on the activation of RTK's and PTK's relate primarily to the effects of exogenously added oxidants. The significance of such actions in specific growth factor- or cytokine-mediated signalling is less clear, although this does not exclude the possibility that, under certain (pathological?) conditions associated with oxidative stress, ROS may directly activate cell surface receptors.

Lipid Metabolism

PLCγ produces inositol 1,4,5-triphosphate [Ins(1,4,5)P_3] which mobilises Ca^{2+} from the intracellular pool to the cytosol, and Diacylglycerol (DAG) which activates PKC.

H_2O_2 induces the phosphorylation of PLCγ together with the production of inositol phosphates (76-78), and oxidants also have been shown to activate phospholipase A2 (PLA2) (79,80) and phospholipase D (PLD)(81,82). Whether phospholipases are direct target of free radicals or act in concert with activated PTK's remains unclear. Activated RTK's can stimulate PI3-kinase, which in turn activates several protein kinases such as Akt , PKCη and PKCε. Furthermore, oxidant mediated Ca^{2+} mobilisation (mediated by Ins(1,4,5)P_3) is blocked by herbimucin A, a PTK inhibitor (83) and partly suppressed in mutant cells that do not express Syk or Lyn(84). This suggests that the Ins(1,4,5)P_3 is activated through the activation of upstream RTK's.

Ca^{2+} signalling

Ins(1,4,5)P_3 generated by PLCγ binds to the Ins(1,4,5)P_3 receptor/ Ca^{2+} channel on the endoplasmatic reticulum, thereby elevating the cytosolic Ca^{2+} level. After the mobilisation of Ca^{2+} from the intracellular pool, there is an influx from the extracellular space.

Oxidative stress increases the cellular Ca^{2+} levels consistent with the production of inositol phosphates. Additionally, both $O_2\cdot$ (85-887) and H_2O_2 (88) have been shown to inhibit the activity of ATP-dependent Ca^{2+} of the SR, which would result in passive diffusion of SR Ca2+ into the cytosol. These effects may be more important in oxidative stress responses (89) than in receptor-mediated signalling by growth factors and/or cytokines.

Small GTP-ases

The superfamily of the Ras-like small GTPases comprises a large family of 20-25kDa proteins, which bind guanine nucleotides very closely and cycle between an inactive GDP-bound state and the active GTP-bound configuration. An upstream signal stimulates the exchange of GDP for GTP and is catalysed by Guanosine exchange factors (GEFs). The GTP-bound form is converted back to the GDP bound form due to intrinsic GTPase activity and is catalysed by GTPase activating proteins (GAPs). Based on sequence and apparent functional homology, the superfamily of Ras-related proteins can be subdivided into the Ras, Rac/Rho, Rab, Arf, Sar, Ran and Rad subfamilies. The Ras subfamily consists of Ras (with 3 isoforms H-, K- and N-Ras), R-Ras, TC21 and Rap (4 isoforms: Rap1A,B and Rap2A,B). Ras is the archetype of the family of small GTPase (for review cfr Bos 90,91), and was originally found as the oncogene in animal tumour viruses. Subsequently 15-30% of all human tumours have been found to contain an activated allele of the Ras gene. Although Ras is situated upstream is signalling pathways leading to intracellular ROS production [constutive active Ras generates free radicals in fibroblasts whereas dominant negative Ras inhibits receptor induced ROS production (64)], Ras itself was also shown to be activated by oxidative stress (92).

Serine/Threonine Kinases and Phosphatases.

The MAPKs comprise a large family of PKs that include ERK1 (p44MAPK)/ERK2 (p42MAPK), JNKs (also known as the stress-activated PKs), and p38 MAPKs. Because the MAPK pathways mediate both mitogen- and stress-activated signals, there has been significant interest in the redox regulation of these pathways. A number of groups (93-98) have

demonstrated the ability of exogenous oxidants to activate the ERK MAPK pathway. The mechanism(s) for this effect is unclear, and the precise molecular target(s) is unknown. Some studies suggest that ROS-mediated ERK activation may be an upstream event at the level of growth factor receptors (95), Src kinases (99), and/or p21Ras (100). Another potential mechanism for this effect may be oxidant-induced inactivation of protein tyrosine phosphatases (PTPs) (101). Most reports imply JNK and p38, but the mitogenic ERK MAPK pathway and other members of the MAPK family have also been implicated as potential targets of ROS. Big MAPK-1 (BMK-1) appears to be much more sensitive than ERK1/ERK2 to H_2O_2 in several cell lines tested and suggests a potentially important role for BMK-1 as a redox-sensitive kinase (102). Although not directly related to the MAPK family, p66shc, a splice variant of the p52shc/p46shc protein involved in mitogenic signalling from activated receptors to p21Ras, was found to be a target of H_2O_2 and responsible for mediating stress apoptotic responses (103).

2.3.5 Redox regulation of Trancription Factors

Oxidative radical stress induces the expression of many genes such as c-fos, c-jun, c-myc and a gene for haeme oxygenase. Such expression is mediated by transcription factors in response to the activation of upstream cellular signalling pathways. Indeed, treatment of cells with H2O2 induces the activation of the transcription factor activator protein-1 (AP-1) and nuclear factor κB (NF-κB) in some cell types. However, it should be noted that many transcription factors are directly regulated by redox in the opposite manner.

NF-κB

NF-κB is a transcription factor that regulates the expression of a number of genes involved in immune and inflammatory responses and has long been considered oxidant responsive (104,105). Free radicals activate NF-κB in response to the activation of upstream cellular signalling pathways. However, to be functional, for NF-κB the DNA binding region need to be kept in a reduced state. The DNA-binding activity of NF-κB is inactivated by treatment with NEM and diamide. Conversely, DTT and β-mercaptoethananol enhance its DNA binding. Moreover, a critical step in NF-B activation is the phosphorylation of IB. The upstream IB kinases that phosphorylate IB are also subject to oxidative stress downmodulating NF-κB activation. Li and Karin (106) found that this redox-regulated effect is observed to occur downstream from the IB kinases, at the level of ubiquitination and/or degradation of IB.

Recent reviews (107, 108) on this subject have therefore emphasised the dual regulation of NF-κB by oxidative stress and the importance of the recognition that a redox-dependent activation of NF-B is cell and stimulus specific as opposed to the concept that oxidative stress is a common mediator of diverse NF-B activators.

AP-1

The oncogene products Jun and Fos form AP-1 by hetero (Jun/Fos) or homo (Jun/Jun) dimerisation, through their leucine-zipper structure located at the C terminal region. However, unlike NF-B, however, AP-1 appears also to be activated by antioxidants (109, 110). In vitro experiments suggest that a single cysteine residue in the highly conserved tri-amino acid sequence Lys-Cys-Arg of the DNA binding domain of Fos and Jun proteins confers redox sensitivity to AP-1 (111). The reduced state of this cysteine residue is essential for its DNA-binding and transformation activity. The modification of this residue by a sulphydryl-modifying agent such as N-ethylmaleimide (NEM) reduces its activity, whereas treatment with reductants such as dithiothreitol (DTT) enhances DNA binding (111). So oxidative stress implicates downregulatation of AP-1 activity. Further evidence suggests that the redox regulation of AP-1 DNA binding is facilitated by the reducing activity of redox factor-1 (Ref-1) protein that may act directly on this critical cysteine residue (112).

Other transcription Factors

Analogous to AP-1 the conformation of the DNA binding of Myb (113), Sp-1 (114) and egr-1 (115) seem to be regulated by similar redox mechanisms as AP-1 with a cystein residue in a region of the helix-turn-helix of which the reduced state is essential.

The tumor suppressor p53 receives much more complicated redox regulation through many cysteine residues (116). This factor binds to Zn through many cysteine residues and act as a transcription factor inducing cell cycle arrest or apoptosis. While the activity of p53 is modulated by copper through a redox mechanism, treatment with NEM or diamide disrupts wild-type p53 conformation and inhibits its DNA binding activity.

Table 2: Signalling molecules regulated by redox in the cells

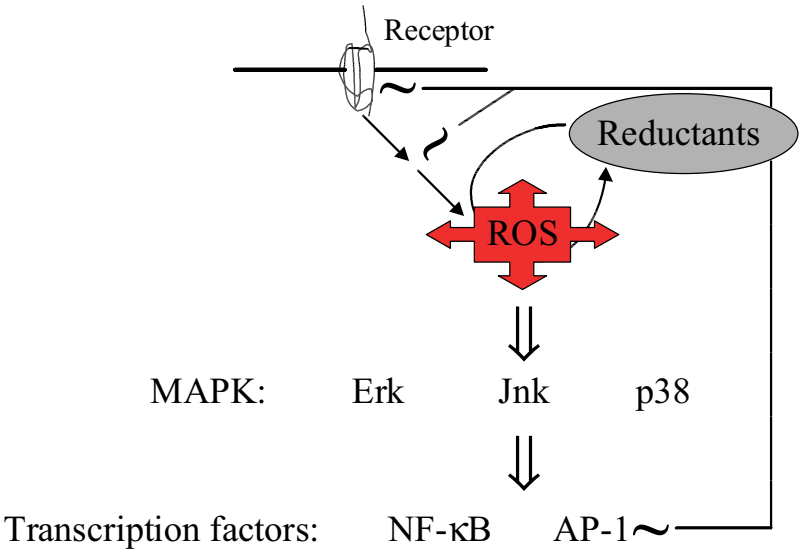
Signalling molecule	Effect of oxidants
1. Prot Tyrosine kinase EGF receptor, insulin receptor, PDGF receptor, Src, Lck, Fyn, ZAP-70, Syk, Lyn, Fgr, Hck, Btk, Ltk	Activation
2. Prot Tyrosine phosphatase	Inactivation
3. Lipid signalling PLC, PLD, PLA_2,PI 3 kinase	Activation
4. Ca2+ signalling Ins(1,4,5) receptor, Ca^{2+}ATPase	Activation
5. small G protein Ras	Activation
6. Protein serine/threonine kinase MAPK, JNK, p38, BMK1, Akt, S6 kinase, PKC	Activation Activation/ inactivation
7. Protein serine/threonine phosphatase PP1, PP2A, calcineurin	Inactivation
8. Transcription factors AP-1(c-fos,c-jun), NFκB, rel, USF,TTF-1, GR, BPV1E2, NFI, Myb, NF-Y, p53, PEBP2/AML, Oct2, Egr-1, BZLF1, Ets, GABP, ATF, CREB, TTF, Ku	Activation/ inactivation

2.4 Conclusion

The information obtained over the last decade has shown that (*1*) a large number of growth factors and cytokines are capable of generating ROS in a number of different cell types, (*2)* inhibitors of ROS-generating enzymatic systems block specific growth factor- and/or cytokine-activated signalling events or physiological effects, and *3*/ exogenous oxidants activate the same cytokine- and/or growth factor-mediated signalling pathway or produces the same physiological effects. This has led to the hypothesis that ROS act as vital mediators in signal transduction. Moreover, the intrinsic characteristics of free radicals make them ideal intracellular messengers.

Anti-oxidant systems from their part are crucial for maintaining a reduced state of most signalling proteins and transcription factors, critical for optimal functioning. Depletion of anti-oxidant systems will therefore lead to cell arrest or apoptosis. On the other hand supplementation of anti-oxidants will scavenge intracellular ROS, thereby counteracting their function.

Figure3: proposed role for ROS as mediator in cellular signaling



3. Oxidative stress in RA

3.1 Evidence of oxidative stress in RA.

It has long been appreciated that oxidative stress plays an important role in RA. RA patients have low concentrations of protective antioxidants: vitamins A, E, C and activities of GSH-Px, and SOD (148,149). Additionally, high levels of the metabolic products of ROS in blood and synovial tissue. RA patients were shown to have high synovial fluid and serum levels of the lipid peroxidation (148-150). Indirect evidence for a role of ROS in cartilage degradation comes from the presence of cartilage-derived peroxidation products (151), a nitrous type II collagen peptide (152), modified low-density lipoprotein (LDL) (153) and oxidized IgG (154) in the serum and urine of patients with arthritis. Since nitrotyrosine, nitrated proteins and oxidized LDL (ox-LDL) have been found to be accumulated in cartilage of arthritic patients, a direct implication of ROS in chronic arthritis has been suggested. Furthermore, a low antioxidant status was shown to be a risk factor for developing RA, according to a case control study within a Finnish cohort of 1419 adult men and women (155). A prospective case-control study within blood donors also showed significant lower β-carotene in patients who developed RA (156).

Treatment with antioxidants was shown to have beneficial effects in animal models of arthritis. High dosis of vitamin C (157), vitamin E (158) and N-acetylcysteine (159) had anti-arthritic effects related to their anti-oxidative potential. Other candidate antioxidants have been identified with potential benefit including selenium, zinc, niacinamine, manganese, β-carotene and bioflavonoids. Nevertheless, clinical trials with RA patients have been disappointing. A randomized, double-blind comparisement of high-dose vitamin E, the most powerfull naturally

occurring lipid-soluble antioxidant, did not show any anti-inflammatory effect as compared to placebo (160). Two placebo-controlled trials with selenium-enriched yeast did not show any clinical benefit (161,162).

3.2 Functional implications of ROS in synovial T lymphocytes

T lymphocytes derived from the synovial fluid from RA patients were shown to suffer from severe oxidative stress, with depletion of intracellular glutathione (5) and upregulation of thioredoxin (163). These findings were thought to underly the hyporesponsive state of SF T cells as demonstrated by the hyporesponsiveness to mitogenic stimuli of SF T cells as well as defective signalling events (e.g. the perturbed membrane localisation of LAT, thus preventing TCR-induced LAT phosphorylation and subsequent recruitment of phospholipase C-γ1 into signalling complexes (5,6). In vitro replenishment with the anti-oxidant NAC restored GSH equilibrium, concomittant with recovery of TCR-dependent LAT phosphorylation and partial rescue of SF T cell hyporesponsiveness, while treatment of T cells from healthy volunteers with DL-buthione(S,R)-sulfoximine (BSO) resulted in depletion of intracellular GSH and mimicked the observed signalling defects (5,6).

3.3 Sources of ROS

Several sources of ROS in the synovial joint that could lead to the disturbed redox homeostasis in SF T lymphocytes have been proposed. These include exposure to free radicals liberated by activated phagocytic cells at the site of inflammation (164), ischemia/reperfusion compromised oxygen radical tension in the inflamed joint (165), and generation of hydroxyl radicals by Fe^{2+} released from dying cells (166).

Extracellular ROS

The major cause of disturbed redox homeostasis in RA has been attributed to chronic exposure of the SF T lymphocytes to oxygen radicals liberated by activated phagocytic cells at the site of inflammation (164). Activated leukocytes can rapidly consume extra oxygen (oxidative burst) with the production of free radicals at their plasma membrane, which are released in the environment. Besides the activated macrophages that may function as a source of free radicals, additional mechanisms have been postulated to contribute to the increased production of ROS in inflamed joints.

Ischemia/reperfusion

During movement, there is an increase of intra-articular pressure (which is more severe in inflamed joints) and the synovial blood flow is compromised, generating a fall in synovial O_2-tension (167,168). This relative synovial hypoxia decelerates the mitochondrial oxidative phosphorylation, and gives rise to the concentration of ATP metabolites such as adenosine, inosine and hypoxanthine in the SF, which are substrates of hypoxanthine dehydrogenase. But under ischemic conditions hypoxanthine dehydrogenase is also converted to hypoxanthine oxidase (XO) by –SH oxidation and partial proteolysis. After exercise, when oxygen becomes available through reperfusion, XO oxidises the accumulated hypoxanthine to uric acid with the production of ROS (169).

Catalytic iron

In the rheumatoid synovium high levels of iron are present. This 'catalytic' iron is probably derived from dead cells in part from traumatic microbleeding. In the presence of Fe^{2+}, superoxide and H_2O_2 lead to the formation of the more toxic hydroxyl radical. The possible contribution of iron to the inflammatory process was shown by the exacerbation of arthritis after iron-dextran infusion, which was associated with increased oxidative damage to lipids and proteins (166)

3.3 Consequences of oxidative stress

Chronic oxidative stress leads to diminished T cell response upon antigenic stimulation as was demonstrated not only in synovial T cells (5,6), but also in T lymphocytes isolated from patients

with AIDS (170) and in ageing T lymphocytes(171). Moreover, as reviewed above, free radicals themselves can serve as intracellular second messengers. In particular, the pro-inflammatory transcription factor NF-κB is activated following treatment with H_2O_2 (172). This results in pro-inflammatory gene expression, induction of TNF-α, IL-1, NO-synthetase, and upregulation of ICAM and VCAM, all of which will contribute to the rheumatic inflammation (173, 174).

4. Scope of this thesis

The studies presented in this thesis were designed to elucidate the mechanisms that lead to oxidative stress in synovial T cells in RA, and to investigate whether altering the redox balance through anti-oxidants can modify the course of the disease. **In chapter 2**, we describe a new method for immunohistochemical detection of free radicals on fixed frozen tissues. We show that T lymphocytes that reside within the synovial tissue are characterised by the abundant production of intracellular ROS, suggesting that the oxidative stress of SF T cells originate from intracellular rather then extracellular sources. In **chapter 3** we focus on the molecular basis of the oxidative stress: we delineate a signaling pathway in T cells downstream from Ras, leading to the generation of ROS, while Rap1 (another small GTPase) signaling is required for downregulating agonist and Ras-induced ROS production. Moreover in SF T cells from patients with RA we find constitutive activation of Ras and a block of Rap1, consistent with activation of the signaling pathway leading to chronic intracellular ROS production. As we show in **chapter 4**, the inhibition of Rap1 block in T cells is induced through cell-cell contact with synovial macrophages. Blockade of synovial monocyte signaling to T cell CD28 with CTLA-4-Ig fusion protein relieved Rap1 inhibition and subsequent ROS production. Additionally, introduction of active RapV12 into T cells prevented SF monocyte-induced T cell ROS production. Also, coincubation of T cells with stimulating anti-CD28 antibody and inflammatory cytokines leads to a synergistic increase in T cell ROS production.
In **chapter 5 and 6** we investigate whether supplementation of oxidants can alter the course of rheumatoid arthritis. In a large double-blind placebo-controlled trial, we find no beneficial effect of a nutrient supplement containing most anti-oxidant substances (**chapter 5**). The clinical effects of intravenously administered NAC are described in **chapter 6**, as well as the effects on the functional properties of peripheral blood and synovial fluid T cells.

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