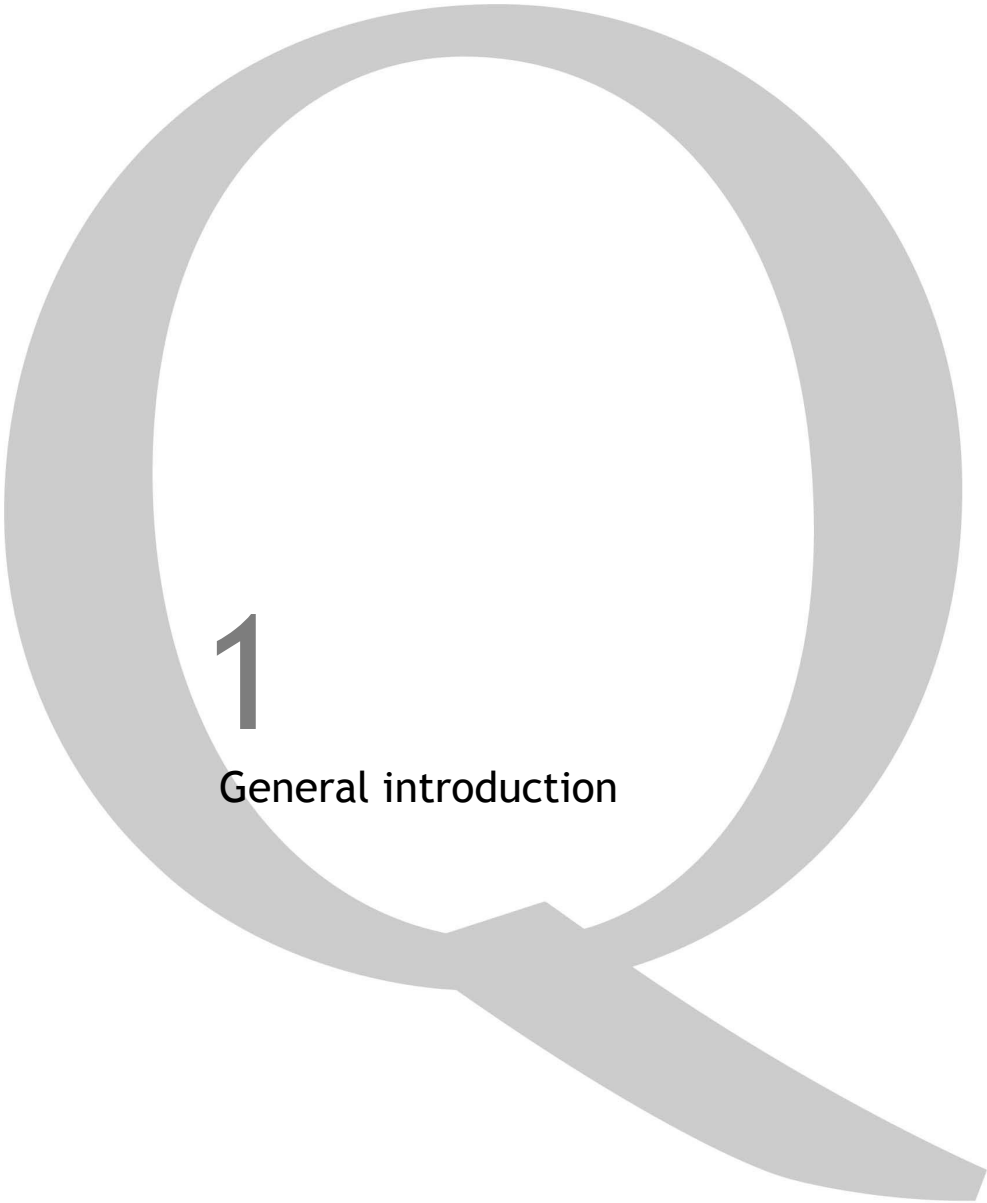

1

General introduction

Introduction

1.1 Free radicals and reactive species

1.1.1 Origin and terminology

Free radicals are reactive molecules due to the presence of one or more unpaired electron(s). They are formed in the human body either as an essential mediator in vital processes including neurotransmission and inflammatory reactions, or as a byproduct that does not have a role in the actual process. In aerobic life forms, the reduction of oxygen is of special interest. This reduction comprises binding of most of the oxygen to hydrogen to give water, a process involved in the oxidative phosphorylation. However, a small part of the oxygen (approximately 1-3%) is only partly reduced during this redox reaction (1). As a result, free radicals or reactive species, that can either oxidize other compounds or easily form radicals, will arise. These partly reduced forms of oxygen are collectively described as reactive oxygen species (ROS). Similarly, reactive nitrogen species (RNS) can be produced. Physiological important ROS include superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), ozone (O_3) and hypochlorous acid (HOCl). Examples of physiological important RNS are nitric oxide ($NO^{\bullet}$) and peroxynitrite ($ONOO^-$). An overview of reactive oxygen and nitrogen species is given in Table 1.1 (2). It should be noted that not all ROS and RNS are equally reactive. Some compounds, such as H_2O_2, $O_2^{\bullet -}$ and $NO^{\bullet}$ react *in vivo* relatively selectively with only a few biological molecules, whereas for example the radical $OH^{\bullet}$ is a very reactive ROS that will react instantaneously with virtually any molecule it encounters (3). The reactivity of the other ROS and RNS lies in between these extremes (4-6). Another difference between various ROS comprises the site of their reactivity; free radicals will react almost instantaneously at the site of their formation, whereas non-radical ROS such as H_2O_2 might also pass biological membranes and in that way spread their reactivity and possible toxicity.

1.1.2 Beneficial effects

As mentioned above, in the human body ROS and RNS are produced that display several crucial physiological functions, including smooth muscle relaxation, metabolism of xenobiotics and the respiratory burst to kill invading micro-organisms (7-10) .

Table 1.1 Typical physiological reactive oxygen and nitrogen species.

Reactive Oxygen Species (ROS)	
Radicals	**Non-radicals**
Superoxide anion, $O_2^{\bullet-}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $OH^{\bullet}$	Hypochlorous acid, HOCl
Peroxyl, $RO_2^{\bullet}$	Ozone, O_3
Alloxyl, $RO^{\bullet}$	
Reactive Nitrogen Species (RNS)	
Radicals	**Non-radicals**
Nitric oxide, $NO^{\bullet}$	Nitrous acid, HNO_2
Nitrogen dioxide, $NO_2^{\bullet}$	Nitrosyl cation, NO^+
	Nitroxyl anion, NO^-
	Peroxynitrite, $ONOO^-$
	Alkyl peroxynitrites, ROONO

Smooth muscle vasodilatation depends on the release of a relaxing factor from the endothelium of blood vessels. This factor, initially referred to as the endothelium-derived relaxing factor (EDRF), appeared to be $NO^{\bullet}$ (11). This radical is produced by nitric oxide synthases (NOS) out of *l*-arginine. $NO^{\bullet}$ will activate guanylate cyclase that forms cyclic guanosine monophosphate (cGMP), which may lead to muscle relaxation (10,12,13). Muscle relaxation can be stopped by the reaction of $NO^{\bullet}$ with $O_2^{\bullet-}$, that is formed locally in the endothelium of the blood vessels (14).

Numerous compounds are metabolized in the liver into more polar compounds by cytochrome P-450 (9). The reactive oxygen generated in the active site of this enzyme can oxidize virtually any endogenous compound or xenobiotic, including chemically very inert compounds as benzene (9,15).

During infections, ROS such as $O_2^{\bullet-}$ and HOCl are used as lethal weapon to kill invading micro-organisms. The explosive production of ROS by phagocytic leukocytes (i.e. neutrophils, eosinophils, monocytes and macrophages) is called the respiratory burst (8,16). Important enzymes that are involved in this inflammatory reaction include nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, the inducible form of NOS and myeloperoxidase.

NADPH oxidase is dormant in resting phagocytic cells but comes into action when the cell is activated by invading micro-organisms (17). This enzyme initiates the oxidant generation of the respiratory burst by forming $O_2^{\bullet-}$. The inducible form of NOS is expressed on phagocytes and, upon stimulation, will form $NO^{\bullet}$ (18-21). $O_2^{\bullet-}$ and $NO^{\bullet}$ themselves are not very reactive towards micro-organisms. On the contrary, the product formed out

of these radicals, i.e. $ONOO^-$, is very cytotoxic (22). Furthermore, the bactericidal activities of the phagocytes are boosted by the formation of the highly reactive HOCl out of H_2O_2 and chloride, a reaction catalyzed by the enzyme myeloperoxidase (23,24). The H_2O_2 necessary for this reaction is formed by the dismutation of superoxide anion, either spontaneously or catalyzed by the enzyme superoxide dismutase (25,26).

1.1.3 Damaging effects

ROS and RNS react readily with practically all bio-molecules, including DNA, RNA, proteins, carbohydrates and lipids, thereby damaging the attacked molecule (27). The reaction with these molecules often starts with the subtraction of a hydrogen atom from the attacked molecule, thereby converting the unpaired electron into a more stable electron-pair. Alternatively, an electron instead of a hydrogen atom might be transferred. From an electrochemical point of view, the hydrogen or electron donating molecule is oxidized. Consequently, free radicals and reactive species are often called (pro)oxidants.

An important target of oxidation by ROS and RNS are the poly-unsaturated fatty acids (PUFAs) present for example in the cell membrane (28). Initiation of this reaction involves the subtraction of a hydrogen atom from the attacked PUFA, thereby leaving an unpaired electron on the lipid. This newly-formed lipid radical undergoes molecular rearrangement to increase its stability (29) and will then rapidly react with oxygen, thereby creating a peroxyl radical. Subsequently, this peroxyl radical will create a lipid hydroperoxide as well as a new lipid radical by subtracting a hydrogen atom from a second PUFA. After molecular rearrangement and the reaction with oxygen and a third PUFA, a second lipid hydroperoxide and a third peroxyl radical are generated. Propagation of this chain reaction, referred to as lipid peroxidation, takes place by continuously passing the unpaired electron from one molecule to another (28). Termination of this chain reaction may occur upon (i) the consumption of one of the two reactants, i.e. the PUFAs or the oxygen, (ii) the formation of a relatively unreactive radical or (iii) the reaction of two radicals that will combine to form a non-radical pair. Products formed during lipid peroxidation include 4-hydroxy-2-alkenals and the three-carbon compound malondialdehyde (MDA) (28). By reacting with DNA bases, MDA can cause mutagenic lesions that may be involved in the pathology of various diseases (30). MDA is widely used as an index of the occurrence of lipid peroxidation in the biological science, although its use is debated (28,31,32).

The initiation and propagation of lipid peroxidation are schematically depicted in Figure 1.1 and illustrate how free radicals may damage bio-molecules.

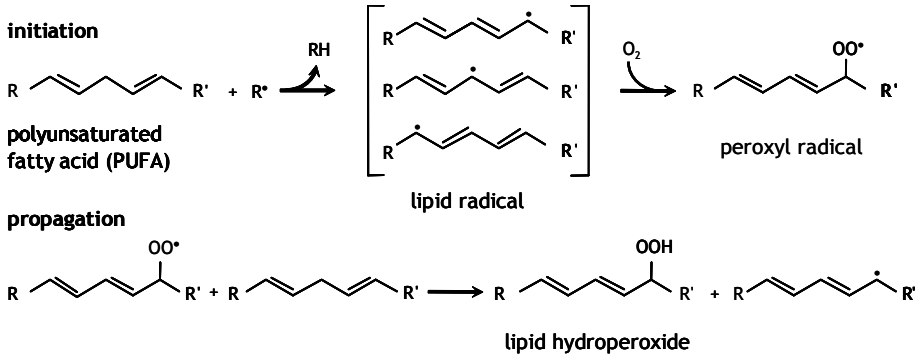

Figure 1.1 Process of lipid peroxidation, started by the radical R•.

Oxidative damage caused by ROS and RNS will lead, among others, to DNA lesions (33-35), function loss of enzymes (36,37), increased cell permeability (38,39), disturbed signaling over the cell (38-40) and eventually even necrotic cell death or apoptosis (27,39-41). Consequently, damage induced by reactive species is often suggested to play a role in the pathophysiology of various diseases, including diabetes (42), cancer (43), chronic obstructive pulmonary disease (44), sarcoidosis (45,46) and idiopathic pulmonary fibrosis (47).

1.1.4 Link between reactive species and inflammation

Besides directly or indirectly damaging various bio-molecules, reactive species are also involved in inflammation. The inflammatory response is designed to recognize, attack and kill invading pathogens (48,49). In vertebrates, this response is subdivided into two parts determined by the speed and specificity of the reaction, i.e. the innate and the adaptive immune response.

The innate immune response mediates the direct protection against infections, either by preventing its occurrence (via epithelial barriers) or by eliminating microbes (via phagocytes, natural killer cells or the complement system) (49,50). The adaptive immune response does not develop instantaneously and is mediated by lymphocytes and their products. B-lymphocytes will produce antibodies that are capable of counteracting infections and eliminating microbes, whereas T-lymphocytes will destroy the microbes directly (48,49).

To offer maximal protection against invading pathogens, cooperation between the innate and adaptive immunity exists. An example of this

interaction is that maturation of dendritic cells, controlled by the innate response, will lead to migration of these cells to lymphoid organs where they can activate the adaptive response by priming T-lymphocytes (51).

A key factor in both the innate and adaptive immune response is the production of cytokines, i.e. glycoproteins secreted by various immune cells including macrophages, neutrophils and helper T lymphocytes (48,52). Cytokines may exert either pro- or anti-inflammatory activities. One of the most prominent pro-inflammatory cytokines in both inflammatory responses is the tumour necrosis factor alpha (TNFα), produced by e.g. macrophages upon the activation of the transcription factor nuclear factor kappa-B (NF-κB) (53,54).

The production of various cytokines via activation of transcription factors such as NF-κB and activator protein-1 (AP-1) can also be induced by ROS (45,55-57). *In vitro* studies, using both macrophages and alveolar and bronchial epithelial cells, have demonstrated that oxidants can initiate the production of inflammatory mediators like interleukin (IL)-8 and $NO^{\bullet}$ (58). The activation of NF-κB by ROS is mediated by the breakdown of its inhibitor part, i.e. IκBα, that causes the normally inactive transcription factor to become active. This activation appears to be due to the phosphorylation and the subsequent degradation or displacement of IκBα, catalysed by various subforms of the IκBα kinase (IKK) (56,59-61). However, some studies have suggested other plausible mechanisms behind this activation (62).

Contradictory, a few studies have reported that (cytokine-induced) NF-κB activation can also be inhibited by pre-treatment with or simultaneous exposure, either acute or chronic, to a specific ROS, i.e. H_2O_2 (62-65). Inhibition by H_2O_2 could be adaptively, resulting in a reduced ROS-forming activity that is not capable of effective phosphorylation of IκB (63). Alternatively, this inhibition may be associated with a promotion of apoptosis resulting from too high levels of damage, induced by the presence of both elevated ROS and inflammatory cytokines levels (65). This would be in line with the fact that only intermediate levels of oxidative stress are capable of activating NF-κB, whereas high levels will induce apoptosis (66). This apparent paradox can be seen as a biphasic response of NF-κB to oxidative stress.

In summary, it can be stated that, although some studies display ambiguous results, ROS appear to induce NF-κB activation. Different outcomes of studies regarding this induction may be the result of variables including the cell type, culturing conditions, anti-oxidant defence present, the level of oxidative stress or other inducers and time frames involved (65).

As stated previously, an important cytokine induced by ROS via NF-κB is TNFα. On its turn, TNFα itself can also mediate the activation of NF-κB by e.g. increasing the activation of IKKs (61,67,68) as well as the production of ROS (69). The latter occurs via stimulation of the radical production during both the mitochondrial oxidative phosphorylation (59,70,71) and the inflammatory burst (69,72,73). Furthermore, TNFα can also enhance the transcription of other cytokines, including IL-8 (61,74). This feed forward mechanism, depicted in Figure 1.2, amplifies both the TNFα- and the NF-κB-mediated effects. Final result of this amplification might be cytotoxic levels of cytokines and ROS, resulting in apoptosis.

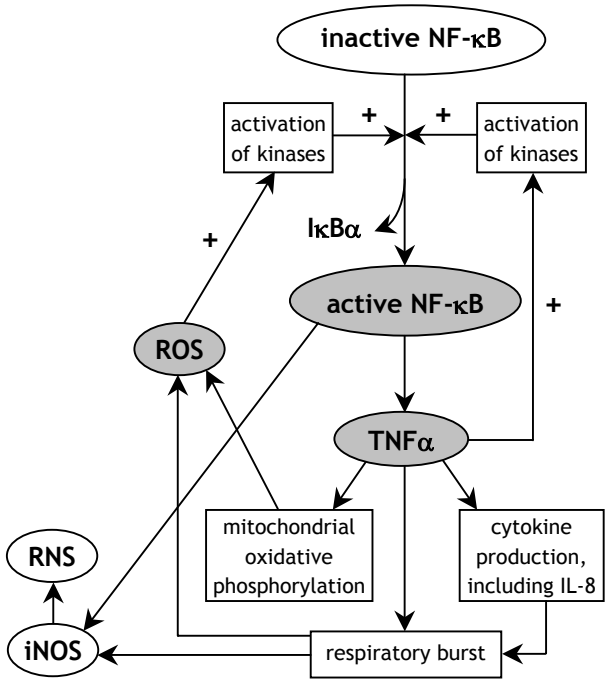

Figure 1.2 Interplay between ROS and inflammation. NF-κB=nuclear factor kappa-B; ROS=reactive oxygen species; TNFα=tumour necrosis factor alpha; RNS=reactive nitrogen species; IL-8=interleukin-8; iNOS=inducible nitric oxide synthase.

1.2 Antioxidants

Fortunately, the human body comprises an elaborate antioxidant defense system to protect cellular compounds from damage induced by free radicals, ROS and other reactive species. An antioxidant has been defined as “any

substance that, when present in low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate" (75,76). The antioxidants that react directly with radicals or other reactive species to prevent cellular compounds from becoming oxidized, can be subdivided into enzymatic and non-enzymatic antioxidants (Table 1.2).

Table 1.2 Enzymatic and non-enzymatic antioxidants.

Enzymatic antioxidants	
Enzyme	**Reaction**
Superoxide dismutases (SOD)	$2\ O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$
Catalases	$2\ H_2O_2 \rightarrow O_2 + 2\ H_2O$
Glutathione peroxidases (GPx)	$2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O$
	$2\ GSH + ROOH \rightarrow GSSG + ROH + H_2O$
Non-enzymatic antioxidants	
Hydrophilic	**Hydrophobic**
Glutathione (GSH)	α-Tocopherol (vitamin E)
Ascorbate (vitamin C)	Carotenoids
Uric acid	Ubiquinol-10

1.2.1 Enzymatic antioxidants

Enzymatic antioxidants react with reactive species and are subsequent efficiently recycled. In fact, the enzyme functions as a catalyst. Consequently, only small amounts of these enzymes are needed to offer protection. Important antioxidant enzymes are superoxide-dismutases, catalase and glutathione peroxidases (27).

Superoxide dismutases (SOD), the first group of enzymes that was discovered with a free radical as substrate (77), catalyze the dismutation of $O_2^{\bullet-}$ to H_2O_2 and oxygen (O_2). Uncatalyzed, this dismutation of $O_2^{\bullet-}$ is relatively slow due to the required encounter of two $O_2^{\bullet-}$ radicals that is hampered by the fact that both radicals are negatively charged and will therefore repulse each other (78). SOD will considerably increase the rate of this dimutation. There are several forms of SOD that all share the feature of containing a transition metal ion, i.e. copper (Cu^{2+}), manganese (Mn^{2+}), iron (Fe^{3+}) or nickel (Ni^{2+}) in their catalytic center. These transition metal ions are capable of catalyzing electron transfer due to their positive charge and their ability to switch to another stable valence or oxidative state (25,79,80). SOD isoforms are distributed throughout the body, including in the mitochondria and cytosol (77,81-83).

Catalase, an enzyme present in all tissues but mostly in the liver and erythrocytes, catalyses the dismutation of hydrogen peroxide into water and

oxygen (78,84). Uncatalyzed, this dismutation is relatively slow due to the fact that H_2O_2 is a weak reductant. Together with most of the enzymes capable of generating H_2O_2, catalase is predominantly located in the peroxisomes of the cell (78,84). Interestingly, catalase is also present in significant, but small, amounts in the mitochondria (85,86).

The selenium-containing glutathione peroxidases (GPx), enzymes distributed in almost all tissues but predominantly in the liver, catalyze the conversion of H_2O_2 into water (78,87-89). GPx can also reduce other peroxides, including lipid hydroperoxide, into alcohol (78,90). In contrast to the dismutases SOD and catalase, GPx is a reductase that consumes a cofactor, i.e. glutathione (GSH). During the conversion of each molecule of H_2O_2, two molecules of GSH become oxidized into one molecule of glutathione disulphide (GSSG) (88,89). Since the activity of GPx requires a constant availability of GSH, GSSG will be reduced by the enzyme glutathione reductase. Conceivably, the tissue distribution of glutathione reductase is similar to that of GPx (91) In the reduction of GSSG, NDAPH is oxidized and therefore, activity of GPx will lead to energy loss.

1.2.2 Non-enzymatic antioxidants

Non-enzymatic antioxidants can be divided into hydrophilic and hydrophobic antioxidants. Hydrophobic antioxidants include α-tocopherol (vitamin E), carotenoids, and ubiquinol-10 and are mostly present in lipoproteins and membranes. Hydrophilic scavengers include GSH, ascorbate and uric acid. They can predominantly be found in cytosolic, mitochondrial and nuclear aqueous compartments (78). The most important endogenous hydrophilic antioxidants that contribute to the total antioxidant defense are GSH, ascorbic acid and uric acid.

GSH is a versatile antioxidant, present in all tissues in high intracellular concentrations ranging from 1 to 10 mM (92,93). Contradictory, extracellular concentrations are significantly lower, i.e. approximately 1 μM in plasma and 100 μM in epithelial lining fluid (ELF) of the human respiratory tract (94). Synthesis of GSH from glutamic acid, cysteine and glycine occurs in many different cell types and is catalyzed by the enzymes γ-glutamylcysteine synthase and glutathione synthase (95,96). Consequently, GSH is not required in the human diet nor can it significantly be taken up in the gastro-intestinal tract (97). GSH represents at least 90% of total low molecular weight non-protein thiols present in cells (96). Free radical scavenging properties of GSH are due to its thiol moiety and result in the formation of a detoxified reactive species and thiyl radicals ($GS^{\bullet}$). The latter can generate GSSG (27). Furthermore, GSH can also exert antioxidant activities by binding free metals through its cysteine thiol group since this will decrease the reaction among

these compounds and oxygen, i.e. the Fenton reaction, that results in the formation of ROS (96,98).

Ascorbate (vitamin C) is an excellent antioxidant, also present in all tissues in high intracellular concentrations (78). Extracellular concentrations are significantly lower, i.e. approximately 60 μM in plasma and 40 μM in ELF of the human respiratory tract (94). In contrast to glutathione and uric acid, ascorbate cannot be synthesized *in situ* in humans. The human inability to make ascorbic acid is due to a lack of L-gulono-lactone oxidase, which catalyses the final step in the biosynthetic pathway (99-101). Consequently, ascorbate is required in the human diet. (102-114). During its scavenging activities, ascorbate becomes oxidized into dehydroascorbic acid (DHA) (78,95). The conversion of DHA into ascorbic acid is shown to be rapidly. This regeneration of ascorbate out of DHA can occur by GSH, suggesting that GSH can boost the function of ascorbic acid (105,106).

Uric acid is a metabolic endproduct of purine metabolism in humans, produced by the enzyme xanthine oxidoreductase (100,107,108). Additionally, uric acid levels are also influenced by diet and will for example increase with enhanced intake of meat or alcohol (109,110). Uric acid is present in all extracellular fluid compartments, but displays the highest levels in plasma (approximately 400 μM) and the epithelial lining fluid of the respiratory tract (approximately 200 μM) (94,107). Uric acid is proven to be a selective antioxidant that is especially capable of scavenging $OH^{\bullet}$ (111). During these scavenging activities, uric acid becomes oxidized into a urate radical anion that, in the absence of redox-partners such as ascorbate, will subsequently be converted into e.g. allantoin and urea (107).

1.2.3 Antioxidant interplay

In contrast to the anti-oxidative enzymes, non-enzymatic antioxidants act by directly scavenging free radicals. During this process, the antioxidants donate an electron or proton to a radical, thereby forming a relatively stable product out of the scavenged radical. Consequently, the antioxidant itself becomes oxidized during this reaction (112,113). Since only the reduced form of the antioxidant can exert scavenging capacities, the oxidized form of the antioxidant, generated during its protective actions against free radicals, has to be converted back into its reduced status. Moreover, the oxidized form of the antioxidant often comprises a radical that, due to some residual activity of its parent compound, might still cause damage to vital cellular targets (112,113). Therefore, the body contains a distinct network of antioxidants that can chemically reduce each other, thereby diminishing the reactivity of the formed antioxidant radical and regaining the reduced antioxidant for the defense against reactive species. In this way, antioxidants act in synergy to

destroy reactive species (78,114,115). A typical example of this antioxidant interplay is shown in Figure 1.3.

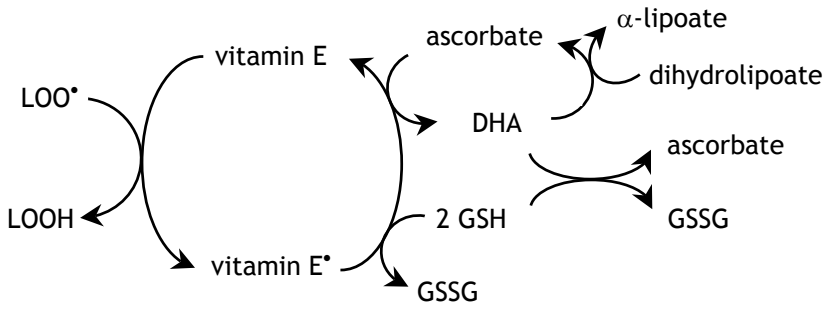

Figure 1.3 Typical example of the antioxidant interplay. LOO•=lipid peroxyl radical; LOOH=lipid hydroperoxide; DHA=dehydroascorbic acid; GSH=reduced glutathione; GSSG=oxidized glutathione.

As depicted in Figure 1.3, the lipid peroxyl radical (LOO•), formed during lipid peroxidation, becomes scavenged preferably by vitamin E. During this reaction, vitamin E becomes oxidized into vitamin E•. Vitamin E• is toxic and as antioxidant useless to the antioxidant defense system. The reaction of vitamin E• with for example GSH results in the reduction of vitamin E• and the concurrently oxidation of GSH into GSSG. In this way, vitamin E is preserved for the defense system at the expense of GSH. Alternatively, ascorbate is also known to regenerate vitamin E out of its radical (116). Recycling GSH or ascorbate for the network may occur by a reaction of their oxidized forms, i.e. GSSG or DHA, with another antioxidant, for example dihydro-lipoate.

This interplay is of special importance since each antioxidant exerts preferentially scavenging activities towards specific free radicals or reactive species at specific compartments. It is known, for example, that LOO• is preferably scavenged by vitamin E while HOCl reacts predominantly with GSH. The interplay between all antioxidants provides an intricate shield that offers optimal defense by the appropriate antioxidant against a particular reactive species at any site throughout the body (78,116).

1.3 Oxidative stress

In normal situations, the endogenous antioxidant network as described in 1.2.3 provides sufficient protection against reactive species such as ROS and RNS (7). However, when an imbalance between the production of and the protection against reactive species occurs in favor of the production, a situation called oxidative stress arises (Figure 1.4).

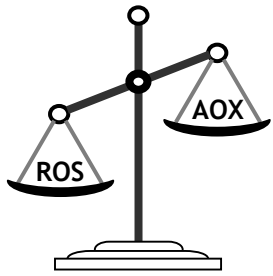

Figure 1.4 Imbalance between production of reactive oxygen species (ROS) and protection against ROS by antioxidants (AOX) results in the occurrence of oxidative stress.

Oxidative stress may result in increased oxidative damage and can be caused either by an overproduction of free radicals and ROS or by an impairment of the endogenous antioxidant defense system (78,117). Due to the intricate antioxidant network, as described earlier, a deficiency in one compound may also affect the efficacy of others. This could result in a greater loss of protection against reactive species than would be expected based on the deficiency of only one antioxidant.

Oxidative stress is associated with the pathophysiology of various diseases including sarcoidosis (46,118,119), idiopathic pulmonary fibrosis (IPF) (45,120,121) and chronic obstructive pulmonary disease (COPD) (44,122). As could be anticipated, due to the link between ROS and inflammation, in most of the diseases associated with oxidative stress elevated inflammation is also implicated.

1.4 Sarcoidosis

Sarcoidosis is a multi-systemic disorder, meaning that it can involve any organ in the body and that its clinical presentation is highly variable. Nevertheless, in 90% of all sarcoidosis cases the lungs are affected (123-125). It is difficult to give a concise definition of sarcoidosis, due to the fact that its exact cause is still unknown, but generally the disease can be described as a systemic, granulomatous and antigen-driven disorder (125-128).

1.4.1 Epidemiology and Prognosis

Sarcoidosis has been known for more than 100 years; it was first described by the dermatologist Hutchinson (1869) and several years later by two other dermatologists, Besnier and Boeck (1888) (124). The disease occurs worldwide and affects both genders and all ages and races. The incidence of the disease varies among different countries over the world. In Scandinavian

countries the incidence is higher compared to more southern countries (123). In the Netherlands and Germany it is estimated that the incidence is approximately 20-25 per 100.000 inhabitants (123).

The clinical symptoms of sarcoidosis patients include, besides fatigue and sometimes other symptoms related to extra-thoracic manifestation, various respiratory symptoms such as dyspnea, coughing and chest pain. Sarcoidosis is a benign disorder with a beneficial prognosis; a large percentage of individuals affected with the disease does not develop clinical symptoms and up to 30% shows spontaneous remission (123). A chronic course, that can result in a significant impairment of lung function, occurs in 10 to 30% of all sarcoidosis patients, whereas the mortality rate is 1 to 6%. In severe clinical cases, sarcoidosis can shift towards another interstitial lung disease, i.e. IPF, due to increased formation of fibrotic tissue in the lungs (129).

1.4.2 Granuloma formation

Main characterization of sarcoidosis is the formation of granuloma, i.e. any small nodular delimited aggregation of mononuclear inflammatory cells. As schematically depicted in Figure 1.5, granuloma formation in sarcoidosis is hallmarked by a highly polarized T-helper 1 (Th1) immune response to unknown endogenous or exogenous antigens (130). This Th1 immune reaction involves the infiltration of $CD4^+$ lymphocytes, following recognition, entrapment and presentation of the unknown exogenous or endogenous antigen by antigen-presenting cells (APC) (129).

The result of the union between $CD4^+$ lymphocytes and antigen-presenting cells is a coordinated release of chemokines and cytokines, predominantly interferon-γ (IFN-γ) and IL-2 respectively. These compounds activate macrophages to release more chemokines and cytokines, predominantly IFN-γ and IL-12, IL18 and TNFα. Subsequently, these chemokines and cytokines will recruit more inflammatory cells (lymphocytes, macrophages and fibroblasts), thereby inducing an amplification pathway that mounts a granulomatous response dominated by IFN-γ, IL-2, IL-12, IL-18 and TNFα (126,131-133). Indeed, the chronic overexpression of TNFα and IFN-γ is suggested to induce persistent inflammation and subsequent tissue damage during sarcoidosis (134,135). Furthermore, the individual capability of a patient to release TNFα is suggested to be linked to the progression of the disease, thereby associating this cytokine to the pathogenesis of sarcoidosis (136).

If the antigen(s) are removed or destroyed, immuno-suppressive cytokines such as transforming growth factor β (TGF-β) will down regulate the immune response and subsequently the granulomatous response will subside; however, persistence of the antigenic stimuli results in chronic disease (128,133,137). Moreover, the development and maintenance of

granuloma may be enhanced in sarcoidosis since sarcoid T-lymphocytes exhibit resistance to apoptosis (138). This is possibly due to a changed expression of molecules which modulate cell survival and death (130). In a small number of patients this inflammatory process will be shifted towards the formation of fibrotic tissue by the overexpression of profibrotic cytokines like TNFα and TGF-ß (133).

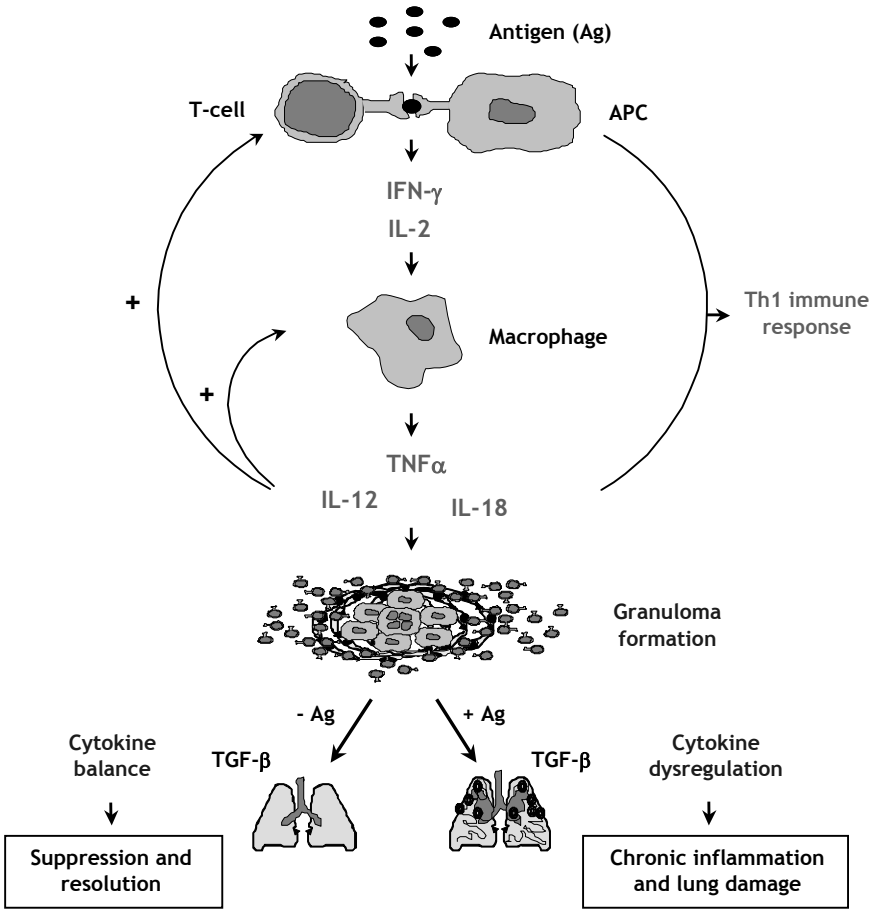

Figure 1.5 A schematic presentation of granuloma formation in sarcoidosis. Ag=antigen; APC=antigen presenting cell; IFN-γ =interferon-gamma; IL=interleukin; Th1=T-helper 1; TNFα=tumour necrosis factor alpha; TGF-β=transforming growth factor beta (adapted from Moller (139)).

1.4.3 Aetiology

Although it is known that a highly polarized Th1 immune response is involved in the pathogenesis of sarcoidosis, the exact aetiology of the disease remains indefinite. However, there is little doubt that environmental and occupational exposure to all kind of triggers may determine the course of the disease (125). Some of the proposed triggers are viruses, mycobacteria, organic agents, including pine tree pollen and cotton dust and inorganic agents like metals (140) and talc (141). Additionally, genetic factors and oxidative stress are suggested to play an important role in the genesis of sarcoidosis (125,126).

Various genetic analyses of families with several members affected by sarcoidosis have revealed that there might be a genetic susceptibility, but that this heritable risk is complex and polygenic (141). One of the genes of which it is suggested that it comprises a polymorphism that could be involved in the aetiology of sarcoidosis is the TNFα gene (136,142,143). Seitzer *et al*. have shown that the more uncommon TNFα2 allele, associated with higher levels of TNFα production, displays a higher frequency in patients with Lofgren syndrome, a benign form of acute sarcoidosis (136). Newly developed molecular genotyping techniques are now being applied to explore more genetic aspects of the disease.

A pivotal role for oxidative stress in the aetiology of sarcoidosis has also been proposed (46,144-146). It has been shown that products of lipid peroxidation, i.e. ethane and 8-isoprostane, are elevated in the exhaled breath condensate of sarcoidosis patients (46,147,148). Additionally, oxidized proteins (149,150) are elevated in the bronchoalveolar lavage fluid (BALF) of sarcoidosis patients. Another consequence of the occurrence of oxidative stress is a reduced redox state, as is reported in the erythrocytes of female sarcoidosis patients (118). Furthermore, the transcription factor NF-κB, of which it is known that it is activated by radical damage, is increased in alveolar macrophages and mononuclear blood cells of active sarcoidosis patients compared to those of healthy controls (119,151). Interestingly, glucocorticoids appeared not to be capable of reducing this increased NF-κB activation in sarcoidosis (119).

1.4.4 Treatment

Conventional treatment is focused on attenuating granuloma formation with antimalarial drugs, that exert anti-inflammatory activities by inhibiting antigen presentation, or with nonspecific anti-inflammatory agents such as glucocortico- steroids, methotrexate, or azathioprine (128). However, these treatments fail to be completely efficacious (152,153). Recently, anti-TNF-α agents such as infliximab have shown some success in sarcoidosis (128,

133,137,154). The design of future therapies depends, among others, on the improved knowledge of the aetiology as well as of the critical immunological processes operative in different stages of the disease (133).

1.5 Antioxidant therapy

Due to their ability to scavenge free radicals and reactive species, thereby reducing oxidative stress and associated damage, various health claims have been made regarding the use of exogenous, dietary antioxidants (27,155). As a result, numerous studies have been performed to examine the possible beneficial health effects of antioxidant supplementation. However, most of these studies have been conducted with healthy volunteers, i.e. people with a sufficient antioxidant shield and no substantial oxidative stress. Therefore, it is not surprising that the outcome of these studies was often rather disappointing (156-160). Additionally, more beneficial health effects of antioxidant supplementation can be expected in patients suffering from a disease that is actually associated with increased levels of oxidative stress, such as sarcoidosis. In these patients, antioxidant levels are impaired and empowering their antioxidant shield via supplementation could, therefore, result in reduction of the elevated oxidative stress present and thus in improvement of their clinical status.

Moreover, free radicals and ROS are also involved in various other processes such as inflammation (45,55), cell-to-cell communication (161), atherosclerotic plaque formation (162,163), angiogenesis (161,164), impairment of receptor functions (165) and DNA lesions (35,164). Therefore, it can be expected that reducing the reactive species load, by strengthening the antioxidant defence with an exogenous antioxidant, will also mitigate these processes. This might be of especial importance in diseases of which the pathology is linked to various of these other processes as well, including sarcoidosis, atherosclerosis and cancer. In such diseases, tackling only one of the underlying processes will most likely not result in an optimal treatment, as can be exemplified by the fact that treatment of sarcoidosis with only immuno-suppressive agents like glucocorticoids fails to be completely efficacious (119). More effect can be expected from treatment with multi-target compounds, such as antioxidants, that are capable of tackling various processes at the same time. Consequently, the use of exogenous antioxidants to support the treatment of these diseases, or maybe even cure them, has gained a lot of interest recently (115). However, most studies regarding the use of antioxidants in chronic diseases have measured the effect of the supplementation on markers of oxidative stress or of one of the other processes mentioned earlier. Only in a relatively few studies the effects of antioxidant supplementation on the clinical status of patients has been

investigated so far. Table 1.3 highlights a selection of these studies, performed in various chronic diseases associated with elevated oxidative stress.

Table 1.3 A selection of clinical studies investigating the health beneficial effects of antioxidant supplementation in chronic diseases.

Anti-oxidant	Dosing regime	Disease	End point(s)	Antioxidant effect(s)	Ref.
NAC	12 weeks, 3x600 mg/day; combined with low maintenance corticosteroids	Pulmonary fibrosis	Pulmonary function tests	Improvement of pulmonary function tests	120
NAC	24 months, 3x600 mg/day; added to standard therapy	IPF	Lung functions including FEV_1 and DLCO	Slower deterioration of lung functions	121
lycopene	1 week, 30 mg/day	Exercise-induced asthma	Post-exercise reduction of lung function FEV_1	Protection against this post-exercise reduction in FEV_1	167
garlic pearls	2 months, 250 mg/day	Essential hypertension patients	Oxidative stress parameters and BP	Reduction of oxidative stress parameters, reduction of BP	168
NAC	72 mg/kg i.v. as a bolus, later 72 mg/kg over 12 h	Pretreatment of cardiac surgery patients	Neutrophil-mediated inflammation and lung injury	Reduced neutrophil influx and elastase activity, possible protection against lung injury	169
α-lipoic acid	3 days, 600 mg/day	Diabetes	Oxidative stress in plasma and NF-κB activity	Reduction of both parameters	170
flavonol-rich cocoa	6 weeks, 444 mg/day	Coronary artery diseases	Vascular function parameters	No effect	171
NAC	10 weeks, 600 mg/day	COPD	Lung functions including FEV_1 and inflammatory parameters including IL-8	No effect on lung functions, reduction of some inflammatory parameters including IL-8	172
polyphenols	5 weeks, 2.66 g/day	COPD	Clinical symptoms and respiratory function tests	No effect	173

NAC=N-acetyl cystein; FEV_1=forced exhaled volume in 1 sec; DLCO=diffusing capacity of the lung for carbon monoxide; BP=blood pressure; NF-κB=nuclear factor kappa-B; COPD=chronic obstructive pulmonary disease; IL-8=interleukin-8.

As can be seen, in pulmonary fibrosis, supplementation for 12 weeks (600 mg/day, combined with a low maintenance prednisolone concentration) with N-acetyl cystein (NAC), an antioxidant and precursor of GSH, caused an improvement of pulmonary function tests (120). More recently, supplementation for 24 months (600 mg 3 times a day, added to standard therapy with prednisone plus azathioprine) with NAC resulted in a significant slower deterioration of two lung functions in IPF, i.e. the diffusing capacity of the lung for carbon monoxide (DLCO) and the forced expiratory volume in one second (FEV_1) (121). In exercise-induced asthma, supplementation for 1 week (30 mg/day) with the antioxidant lycopene prevented post-exercise impairment of FEV_1 in 55% of all patients (166). In essential hypertension

patients, supplementation for 2 months (250 mg/day) with garlic, a compound containing anti-oxidative capacities, caused a mild blood pressure reduction (167).
In cardiac surgery patients, NAC-pretreatment (72 mg/kg i.v. as a bolus, later 72 mg/kg over 12 h) prevented possible lung injury by reducing the neutrophil influx and elastase activity (168). In diabetes, supplementation for 3 days (600 mg/day) with the antioxidant α-lipoic acid decreased the occurring oxidative stress and NF-κB activity but, unfortunately, no clinical parameters were evaluated (169).

Contradictory to the above mentioned studies, some clinical studies failed to show beneficial health effects of antioxidant supplementation. In coronary heart diseases, flavonol-rich cocoa for 6 weeks (444 mg/day) did not modify vascular function (170) and in COPD, either NAC supplementation for 10 weeks (600 mg/daily) nor polyphenol supplementation for 5 weeks (2.66 gr/day) had an effect on lung function (171,172).

Important aspects that should be considered in the use of exogenous antioxidants include their scavenging capacities, their possible place in the antioxidant network and their bio-availability. In other words, the effectiveness of antioxidant therapy relies on whether the appropriate scavenger will attain intracellular concentrations that are high enough to fill the existing defect in the endogenous antioxidant network. Moreover, the possible toxicity of antioxidants, when administered in high levels that exceed their daily intake by far, have to be considered too, as can be derived from the toxic effects of high doses of antioxidant β-carotene found in two clinical trials with heavy smokers and asbestos workers (173-175). Clearly, further research is needed to optimize the use of specific antioxidants in diseases associated with oxidative stress

1.6 The flavonoid quercetin

1.6.1 Origin, structure and intake

Flavonoids are a class of naturally occurring polyphenolic compounds, ubiquitously present in photosynthesising cells (176,177). Over 5000 different naturally occurring flavonoids have already been identified and the list is still growing (178,179). Flavonoids are present in fruits, vegetables, nuts and plant-derived beverages such as tea and wine (180,181). Additionally, many antioxidant supplements as well as herb-containing medicaments contain high doses of flavonoids.

Most flavonoids share a common three-ring structure, depicted in Figure 1.6, of which ring A and B are aromatic and ring C is heterocyclic. The variation in the heterocyclic C ring forms the basis of the division of the

flavonoids in various subclasses, i.e. the flavones, isoflavones, flavonols, flavanols, flavanones, anthocyanidins and chalcones (182).

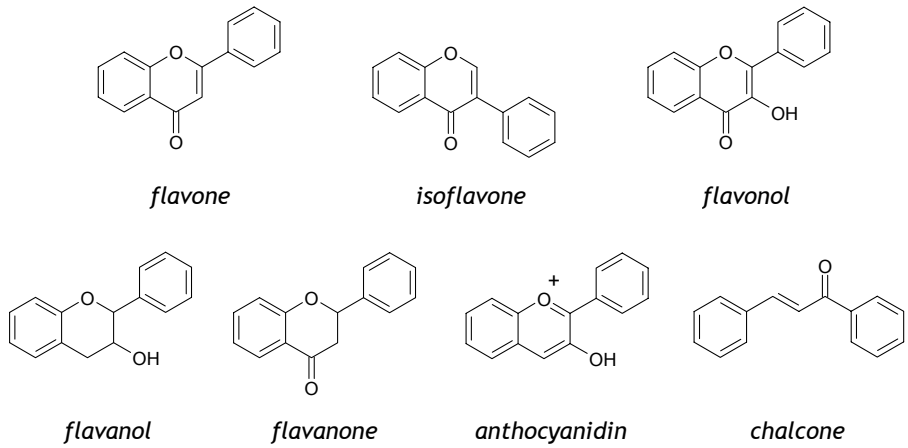

Figure 1.6 Structures of the major classes of flavonoids.

The total amount of flavonoids consumed in the Netherlands is estimated at several hundreds of mg per day (183). The Dutch intake of flavones and flavonols is determined as 23-24 mg per day and quercetin, the main flavonol present in our diet, represents 70% of this intake (183,184). The molecular structure of quercetin is given in Figure 1.7. Quercetin, commonly found in apples, onions and green tea (183), occurs mainly as glycoside, i.e. a sugar group such as glucose, galactose, rhamnose, rutinose or xylose is bound to one of the hydroxyl groups of the flavonol (179,185).

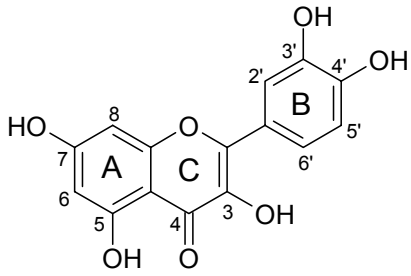

Figure 1.7 Molecular structure of quercetin.

1.6.2 Absorption, metabolism and bio-availability

Because of the hydrophilic character of its glycosides, only quercetin without a sugar group, i.e. the aglycon, was initially suggested to be taken up in the gastro-intestinal tract by passive diffusion (181,186). However, a study with human ileostomy volunteers showed not only that quercetin glycosides can indeed be absorbed in the small intestine, but also that this absorption surpasses that of the aglycon by far, i.e. 52% of the glycosides was absorbed versus 24% of the aglycon (187). Other studies have confirmed that the absorption of quercetin is considerably enhanced by its conjugation with a sugar group (188,189). A possible explanation for this increased absorption, resulting in a higher plasma peak concentration and an increased bio-availability, is the facilitation of glycoside absorption by either deglycosylation (190,191) or carrier-mediated transport (192,193).

Deglycosylation of quercetin glycosides involves β-glucosidases that are capable of liberating the aglycon for passive diffusion (190,191). Especially the intestinal lactase phlorizin hydrolase (LPH) displays a specific activity towards flavonoid glycosides (194) and its role in quercetin glycoside absorption has been confirmed in an *in situ* rat small intestine perfusion model (195). Carrier mediated transport of quercetin glycosides may involve the sodium-dependent glucose transporter-1 (196,197), but a role for other transporter proteins such as the multidrug resistance protein 2 (198) has also been suggested. After their facilitated uptake by means of carrier mediated transport, quercetin glycosides often become hydrolysed by intracellular β-glucosidases (191).

After absorption, quercetin becomes metabolised in various organs including the small intestine, colon, liver and kidney (199). Metabolites formed in the small intestine and liver are mainly the result of phase II metabolism by biotransformation enzymes and therefore include the methylated, sulphated and glucuronidated forms (200,201). Moreover, bacterial ring fission of the aglycon occurs in both the small intestine and colon, resulting in the breakdown of the backbone structure of quercetin and the subsequent formation of smaller phenolics (199,202).

Normally, human quercetin plasma concentrations are in the low nanomolar range, but upon quercetin supplementation they may increase to the high nanomolar or low micromolar range (203,204). Recently, a study regarding the tissue distribution in rats and pigs has shown that, upon quercetin supplementation, the highest accumulation of the flavonoid and its metabolites are found in (rat) lungs and (pig) liver and kidney. Unfortunately, pig lungs were not analysed (205). It has been shown that the half lives of the

quercetin metabolites are rather high, i.e. 11 to 28 hours. This indicates that, upon repeated quercetin supplementation, they could attain a considerable plasma level (188,206).

1.6.3 Beneficial effects

Epidemiological studies

Various epidemiological studies, using data on fruit and vegetable consumption to calculate the mean flavonoid intake, have been performed to provide support for the alleged beneficial health effects of a high intake of flavonoids such as quercetin. A protective effect of flavonole and flavone intake has been found regarding (i) the risk on fatal or non-fatal coronary artery diseases (183,207,208), (ii) the risk on lung cancer (209,210), (iii) the incidence of asthma (210) and (iv) the impairment of pulmonary functions in COPD (211). However, not all epidemiological studies regarding the association between flavonoid intake and the risk on various diseases, including a variety of cancers, were able to confirm this protective effect. Moreover, the alpha-tocopherol-beta-carotene (ATBC)-prevention study showed a borderline positive association between the flavonoid intake and a reduced risk for colorectal cancer (209). A possible explanation for this discrepancy could be due to unsufficient correction for differences in smoking and dietary behavior. Smoking is associated with unhealthy behavior, including a higher intake of energy, alcohol and fat and a lower intake of fruits and vegetables (212,213). Additionally, a high consumption of fruits and vegetables, i.e. important sources of flavonoids, is associated with a more healthy behavior (214,215). Therefore, if not corrected for properly, such differences could result in confounders giving false associations.

Overall, epidemiological studies performed so far support a beneficial role for flavonoids in the lung, but they do not provide conclusive evidence for beneficial health effects of a high flavonoid cq quercetin intake in diseases concerning other organs, including various forms of cancer. Consequently, more accurate epidemiological research is necessary to elucidate the overall beneficial health effects of flavonoids.

In vitro and in vivo studies

Quercetin has been shown to be an excellent *in vitro* antioxidant. Within the flavonoid family, quercetin is the most potent scavenger of ROS, including $O_2^{\bullet -}$ (216,217), and RNS like $NO^{\bullet}$ (218,219) and $ONOO^-$ (220,221). These anti-oxidative capacities of quercetin are attributed to the presence of two antioxidant pharmacophores within the molecule that have the optimal configuration for free radical scavenging, i.e. the catechol group in the B ring and the OH group at position 3 of the AC ring (222). Moreover, quercetin is

suggested to substantially empower the endogenous antioxidant shield due to its contribution to the total plasma antioxidant capacity which is 6.24 times higher than the reference anti-oxidant trolox, whereas for example the contribution of both vitamin C and uric acid virtually equals that of trolox (223).

Until now, very few studies have been performed to examine the *in vivo* anti-oxidative effects of quercetin. *In vivo* quercetin supplementation for 28 days (1 gram a day) in healthy volunteers resulted in a significantly increased plasma quercetin concentration, but did not show any beneficial health effects regarding risk factors for coronary diseases (203). This lack of effect might be explained by the fact that this study was performed in healthy volunteers who display only relatively low levels of oxidative stress and are, therefore, not in need of extra anti-oxidative defense. Another quercetin supplementation in healthy volunteers (11 mg/day for 28 days via a quercetin-rich fruit juice) also increased the plasma quercetin concentration as well as the total plasma antioxidant capacity (224). However, no *in vivo* oxidative stress markers or other parameters to measure *in vivo* health effects were included in this study. Interestingly, the fact that this supplementation did cause a 41% reduction of *ex vivo*-induced oxidative damage ($P<0.07$) might contribute to the rationale for supplementing antioxidants only in situations of elevated oxidative stress.

In vitro, it has been shown that quercetin also possesses anti-inflammatory (225,226), immuno-suppressive (227,228), anti-fibrotic (229), anti-coagulative (230), anti-bacterial (216), anti-atherogenic (231,232), anti-hypertensive (231,233) and anti-proliferative properties (228,234,235). Furthermore, quercetin is reported to directly modulate the gene expression of enzymes involved in biotransformation (236-239) and to inhibit cell proliferation by interacting with estrogen binding sites (240,241). Altogether, these *in vitro* studies indicate that quercetin may exert health beneficial capacities via various damage modulating effects. However, most of these studies have been performed with immortalized or cultured cell lines only and are thus not easy to extrapolate to the *in vivo* human situation. Therefore, whether *in vivo* quercetin supplementation has beneficial health effects remains to be confirmed.

1.6.4 Toxic effects

It is well documented that during its anti-oxidative activities, quercetin becomes oxidized into various oxidation products (Figure 1.8). The two electron oxidation of quercetin yields the oxidation product quercetin-quinone, denoted as QQ, that has four tautomeric forms, i.e. an *ortho*-quinone and three quinonemethides (Figure 1.8) (242-244).

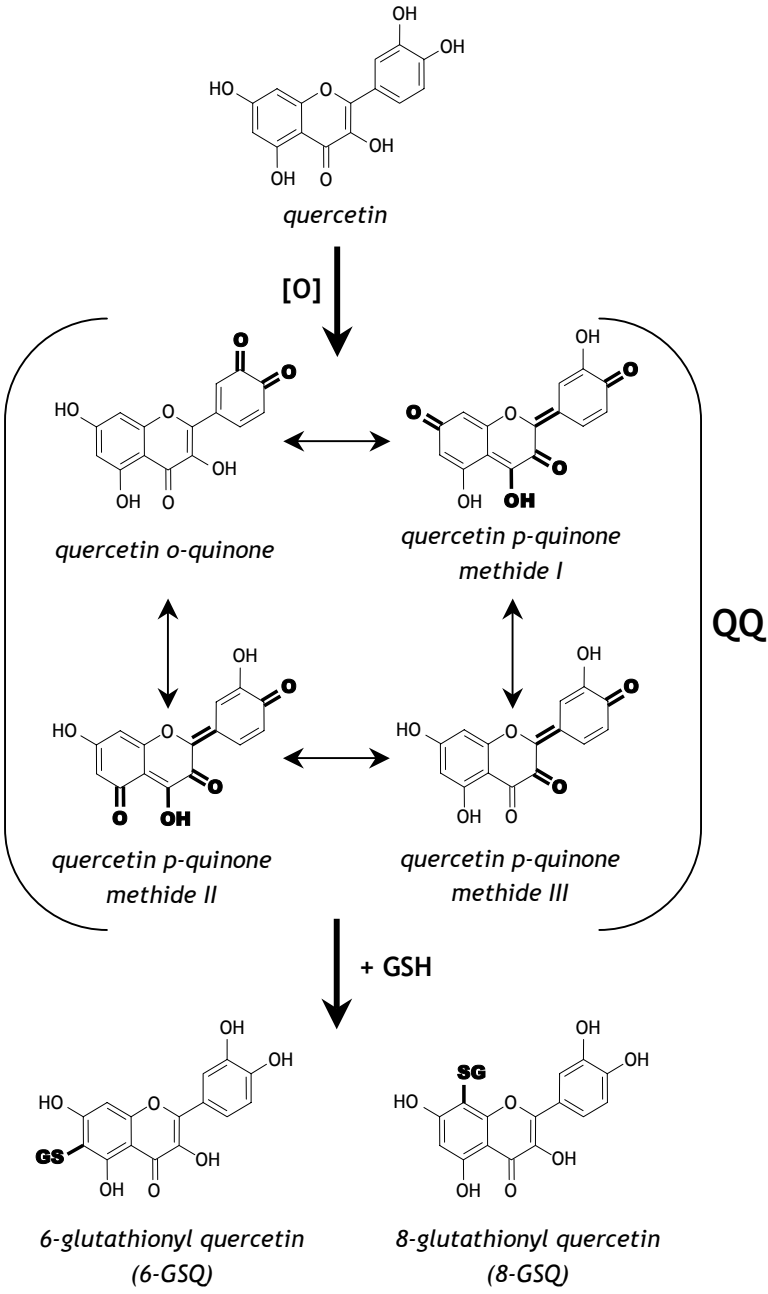

Figure 1.8 Oxidation of quercetin, followed by the possible reaction of its major oxidation product QQ with glutathione (GSH).

It has been well described that oxidation products like semiquinone radicals and quinones display various toxic effects due to their ability of arylating protein thiols (245-248).

Indeed, QQ is very reactive towards thiols and can instantaneously form an adduct with GSH, the most abundant endogenous thiols (243,249). This adduct is called GSQ. When no GSH is present, QQ can react with other thiols, e.g. protein thiols. This reaction may lead to toxic effects such as increased membrane permeability (250) or, as previously shown for other quinones (244,245), altered functioning of enzymes that contain a critical SH-group. Such QQ-induced toxicity has been shown in various *in vitro* studies but the *in vivo* formation, and possible toxicity, of QQ has not been demonstrated yet.

Quercetin has also been reported to display genotoxic effects *in vitro*. Interestingly, these mutagenic effects of quercetin are predominantly shown in bacteria and are suggested to require quinone formation as mediators as well (251-254). In mammalian cells and experimental animals, conflicting data regarding the capacity of the flavonoid to induce DNA lesions and mutations are reported.

On the one hand, induction of chromosomal aberrations and single strand breaks combined with point mutations are shown in respectively hamster ovary (252) and mouse lymphoma cells (255). On the other hand, quercetin supplementation in either mice (256) or rats with aorta restriction (257) could protect against benzo[a]pyrene-induced DNA damage and was suggested, respectively, to protect against the development of lung cancer (256) and to attenuate cardiac hypertrophy (257). Clearly, more research is required to explain the discrepancy observed between the *in vitro* and *in vivo* quercetin genotoxicity studies in order to further elucidate the possible genotoxic effects of the flavonoid for man.

1.7 Aim and outline of the thesis

This thesis focuses on the possible health-beneficial effects of the flavonoid quercetin. Especially the relation between the *in vitro* and *in vivo* behavior of quercetin is explored. Furthermore, the possible use of this flavonoid in sarcoidosis, an inflammatory disease, is examined.

Firstly, the behavior of quercetin during oxidative stress was evaluated *in vitro*. **Chapter 2** describes the protection against lipid peroxidation offered by catechol-containing antioxidants such as quercetin. The effects of its oxidation products, formed during this protection, are discussed. In **chapter 3** the reaction of the main oxidation product of quercetin, i.e. QQ, with thiols is further examined. Especially the reactions of QQ with GSH or with vitamin C are evaluated and compared. To investigate the reaction of QQ with DT-diaphorase (NQO-1), the experiments described in **chapter 4** are performed. The possible role of this enzyme in the protection against QQ-induced toxicity is examined. Since the QQ-toxicity appears to be focused on its reaction with thiols, **chapter 5** focuses on the fate of the adducts formed between QQ and various thiols, including GSH, the most abundant endogenous thiol. The *in vitro* work was concluded by investigating the protective effect of quercetin in relation to the potential toxic effects of its oxidation products in a more integrated cell system, using rat lung epithelial cells (RLEs) as described in **chapter 6**.

Secondly, the *in vitro* and *ex vivo* anti-inflammatory as well as the *in vivo* anti-oxidative effects of quercetin have been examined. **Chapter 7** presents the effects of a four-weeks comprising quercetin intervention on the antioxidant status as well as on the basal and *ex vivo*-induced inflammation in healthy volunteers. The potential toxicity of QQ is also discussed. The *ex vivo* anti-inflammatory effect of quercetin has further been examined in both sarcoidosis patients and healthy controls in **chapter 8**. Moreover, the antioxidant status of both the patients and their matched controls has been quantified.

Thirdly, the *in vivo* anti-oxidative and anti-inflammatory effects of quercetin supplementation in sarcoidosis patients are reported in **chapter 9**. Implications for the use of antioxidant supplementation, with for example quercetin, in the treatment of this disease are given.

Finally, the results and impacts of our findings are summarized in **chapter 10**. Implications for further research are also given.

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