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**Inventory of biomarkers for oxidative stress**

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This investigation has been performed by order and for the account of the DG-RIVM, within the framework of project S/630111/01/AB, 'Reducing uncertainties in the causality between inhalation exposure and health'.

## **Abstract**

This inventory was carried out under the project ‘Reducing uncertainties in the causality between inhalation exposure and health’ prepared in the framework of strategic research at the National Institute for Public Health and the Environment (RIVM). The aim of the project is to validate a model to reduce uncertainties in the causality between inhalation exposure and health effects. The underlying question here is whether health effects due to air pollution are facilitated by an oxidative stress mechanism. This biomarker inventory for oxidative stress is one of the products resulting from this project. Oxidative stress results either due to increased exposure to reactive oxygen species or to the presence of decreased antioxidant defences, resulting in damage to macromolecules. Biomarkers that could be of interest for studying the role of oxidative stress in air pollution-related health effects are carbonyl levels as a measure for oxidized proteins, thiobarbituric acid-reactive substances for total lipid oxidation and 8-hydroxy-deoxyguanosine for oxidative injury to DNA. In the antioxidant response, superoxide dismutase, heme oxygenase, metallothioneins, and thioredoxin reductase and glutathion could be valuable markers.

## Voorwoord

Dit document is de rapportage ‘Inventory of biomarkers for oxidative stress’. Deze rapportage is geschreven als eerste product van het SOR project ‘Vermindering van onzekerheden in causale relaties tussen inhalatoire blootstelling en gezondheidseffecten’, S/630111/01/AB voor de opdrachtgever SOR, de DG-RIVM.

Dit SOR project wordt uitgevoerd in het speerpunt ‘Kwantitatieve Risicoanalyse’ onder het programma 1, ‘Kwantitatieve risicoschatting’. Dit programma heeft als doel de kwantitatieve onzekerheden binnen de risicoschatting te reduceren. De gezondheid van mens en milieu kan namelijk nadelig worden beïnvloed (risico’s) door een aantal externe factoren waaronder fysische, chemische en biologische agentia. Dit specifieke SOR project richt zich op reductie van onzekerheden in de causale relaties tussen inhalatoire blootstelling en gezondheidseffecten met behulp van dierexperimenteel onderzoek.

Blootstelling aan algemene luchtverontreiniging (ozon, fijn stof, binnenhuislucht), maar ook aan emissies bij rampen en branden vormt een voortdurende risicofactor voor de gezondheid. Er zijn vaak grote onzekerheden over de causale relatie tussen deze (acute of chronische) blootstelling en de gezondheidseffecten. Uit epidemiologische studies blijkt echter dat associaties tussen luchtverontreiniging en gezondheidseffecten het meest duidelijk zijn voor bepaalde risico groepen binnen de bevolking, zoals mensen met astma, ‘chronic obstructive pulmonary disease’ (COPD) of cardiovasculaire aandoeningen, alsmede ouderen, jonge kinderen enzovoort. Om het experimenteel onderzoek op het gebied van gezondheidseffecten ten gevolge van luchtverontreiniging te bespoedigen en beter inzicht te verkrijgen in het mechanisme wordt er veelal gebruik gemaakt van diermodellen die tevens representatief zijn voor deze risicogroepen.

Een groeiende risicogroep binnen de samenleving zijn bijvoorbeeld de ouderen.

Leeftijdspecifieke analyses laten statistisch significante associaties zien tussen luchtverontreiniging en sterfte in voornamelijk de ouderen boven de 65 jaar. Ouderen hebben meestal een verzwakte homeostase (de onderling op elkaar afgestemde processen die voor het leven noodzakelijke toestanden constant houden) waardoor ze minder goed in staat zijn adequaat te reageren op oxidatieve stress. Juist oxidatieve stress lijkt een cruciale rol te spelen bij het veroorzaken van gezondheidseffecten ten gevolge van luchtverontreiniging. Echter gedegen inzicht in het werkingsmechanisme ontbreekt.

De centrale **hypothese** binnen het huidige project is derhalve:

*Oxidatieve stress speelt een cruciale rol bij het veroorzaken van de gezondheidseffecten ten gevolge van blootstelling aan luchtverontreiniging.*

De producten van dit project als geheel zullen inzicht geven in de rol welke oxidatieve stress vervult als mechanisme in de gezondheidseffecten ten gevolge van algemene luchtverontreiniging (ozon, fijn stof, binnenhuislucht), maar ook aan emissies van

bijvoorbeeld rampen en branden. De beoogde resultaten van het project liggen dan ook duidelijk op het gebied van verdieping en verbreding van onze kennis op het gebied van externe agentia in de inhalatie toxicologie.

In deze rapportage wordt uiteengezet wat oxidatieve stress is, hoe het ontstaat en wat de kenmerken er van zijn, resulterend in een lijst van zogenaamde biomarkers. Deze inventarisatie is de basis en leidraad voor experimenteel onderzoek wat binnen dit project wordt uitgevoerd.

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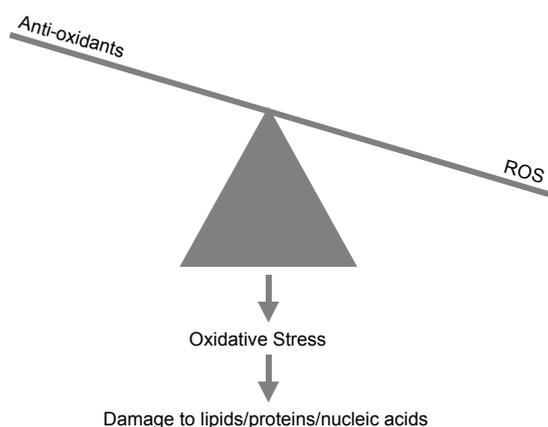
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## Samenvatting

Epidemiologische studies laten consistent associaties zien tussen cardiovasculaire aandoeningen, verminderde long functie, verhoogde ziekenhuis opnamen, sterfte, en luchtverontreiniging. Er is behoefte aan beter inzicht in de causale relatie hiervan. Hiervoor is een grotere kennis van het mechanisme nodig, welke ten grondslag ligt aan deze gezondheidseffecten. Enerzijds maakt dit een meer doelgerichte benadering mogelijk om de meest toxische componenten van lucht verontreiniging te verwijderen. Anderzijds zou het een verlaging kunnen bewerkstelligen van individuele gevoeligheid voor luchtverontreiniging. Recent is oxidatieve stress als mogelijk mechanisme voorgesteld welke ten grondslag ligt aan de toxische effecten van luchtverontreiniging.

### Inventarisatie van parameters voor oxidatieve stress

Oxidatieve stress kan ontstaan wanneer er een verhoging van reactieve zuurstof deeltjes, zogenaamde ROS, of een verlaging in de anti-oxiderende verdediging in een cel ontstaat (Figuur 1). Ten gevolge hiervan ontstaat er oxidatieve schade aan verschillende



*Figuur 1. Schematische weergave van oxidatieve stress welke resulteert in beschadiging van macromoleculen.*

macromoleculen (lipiden, eiwitten en DNA) en kunnen er redox gevoelige signaal routes geactiveerd worden. Door hun reactiviteit zijn ROS zelf zeer moeilijk te meten als indicator voor de ontstane oxidatieve stress. Als indirecte maat voor oxidatieve stress worden er daarom enerzijds de oxidatie producten gemeten en anderzijds de veranderingen in de antioxidant niveaus. Biomarkers welke van belang kunnen zijn in het onderzoek naar de rol van oxidatieve stress in luchtverontreiniging gerelateerde gezondheidseffecten zijn: carbonyl niveaus als maat voor de geoxideerde eiwitten, 'thiobarbituric acid' reactieve substanties als maat voor geoxideerde lipiden, en 8-hydroxy-deoxyguanosine als maat voor oxidatieve schade aan DNA. Wat betreft de antioxidant respons zijn, superoxide dismutase, heem oxygenase, metallothioneins, en thioredoxine reductase en glutathion waardevolle markers voor oxidatieve stress.

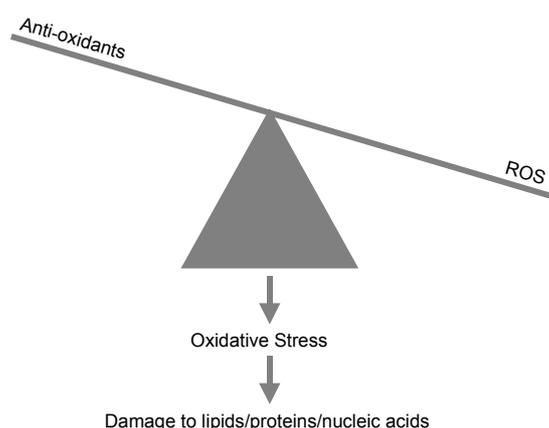
## Summary

Epidemiological studies have shown a clear association between cardiovascular morbidity, decreased lung function, increased hospital admissions, mortality, and airborne concentration of photochemical and particulate pollutants.

A better appreciation of the mechanism underlying air pollution induced health problems would allow a more targeted approach to remove the most toxic components of air pollution, and could possibly provide a means to decrease individual sensitivity to air pollution. In recent research oxidative stress has been identified as a unifying feature underlying the toxic actions of the air pollutants that are causing concern.

### Inventory of oxidative stress parameters

Oxidative stress results from either increased exposure to reactive oxidative species or the presence of decreased antioxidant defences resulting in oxidative damage to lipids, proteins, and DNA (Figure 1). Besides damaging of such macromolecules it also seem to trigger a



*Figure 1. Schematic view of oxidative stress resulting in damage to macromolecules.*

number of redox sensitive signalling pathways. Monitoring this process of oxidative stress is not easy since reactive oxygen species are very reactive particles and therefore difficult to measure. Therefore on one-hand measurements of products, macromolecules damaged by reactive oxygen species, is the most common technique to indirectly measure reactive oxygen species. On the other hand measurement of the primary defense against reactive oxygen species, the endogenous antioxidants, is a way to monitor the situation of oxidative stress. Changes in these antioxidants levels and its balance with reactive oxygen species might give us information about the state of oxidative stress. Biomarkers, which could be of interest for studying the role of oxidative stress in air pollution related health effects, are carbonyl levels as a level for oxidized proteins, thiobarbituric acid-reactive substances for total lipid oxidation, and 8-hydroxy-deoxyguanosine for oxidative injury to DNA. In the antioxidant response, superoxide dismutase, heme oxygenase, metallothioneins, and thioredoxin reductase and glutathion are valuable markers.

# 1. Introduction

There is increasing concern over the adverse effects of air pollution on human health. Epidemiological studies have shown a clear association between cardiovascular morbidity, decreased lung function, increased hospital admissions, mortality, and airborne concentration of photochemical and particulate pollutants.

As with any toxic challenge the obvious solution is to remove, or at least decrease to an acceptable level, the source of trouble. It has been recognised that there is a need to improve our understanding of the impact of air pollution on biological systems as well. A better appreciation of the mechanism underlying air pollution induced health problems would allow a more targeted approach to remove the most toxic components of air pollution, and could possibly provide a means to decrease individual sensitivity to air pollution. Recent research has identified oxidative stress as a unifying feature underlying the toxic actions of the air pollutants that are causing concern. Oxidative stress, resulting from either increased exposure to oxidants or the presence of decreased antioxidant defences, seems to trigger a number of redox sensitive signalling pathways. There is now a strong body of evidence to indicate that the pulmonary inflammatory response that arises following exposure to a pollution episode, is mediated via oxidant signalling pathways. Moreover, it appears likely that an individual sensitivity to pollution is related, in part, to their pulmonary antioxidant defences<sup>2</sup>.

Within RIVM's strategic research programme launched project entitled 'Vermindering van onzekerheden in causale relaties tussen inhalatoire blootstelling en gezondheidseffecten' (S/630111/01/AB) the main hypothesis is that oxidative stress plays a crucial role in health effects caused by air pollution. This hypothesis is tested with use of sensitive models and with the help of new developing techniques, such as genomics. In order to have a good starting position an inventory of biomarkers will give some background about what oxidative stress is in general, what processes might occur in the lung and based on both which biomarkers could be of interest for further research.

## 2. Radicals and ROS

To understand the way 'oxidative stress', as a mechanism, can play a role in the health effects due to air pollution some chemical background should be given concerning radicals and ROS, playing an important role in oxidative stress.

A free radical is by definition any chemical species that contains one or more unpaired electrons. Unpaired electrons alter the chemical reactivity of an atom or molecule by making it more reactive; molecules with an unpaired electron act like electron acceptors and 'steal' electrons from other molecules. When a molecule loses an electron, this is called oxidation. Free radicals are therefore called oxidising agents because they tend to make other molecules donate their electrons. Free radicals that contain oxygen are classified together as reactive oxygen species (ROS). Names as 'active oxygen species', 'reactive oxygen mediators' etcetera also appear in the literature. Well known ROS are superoxide radical anion ( $O_2^{\bullet-}$ ), hydroxyl radical ( $OH^{\bullet}$ ), hydrogen peroxide ( $H_2O_2$ ) (although not a free radical this is considered a ROS).

In the body, ROS are constantly produced and eliminated, since cells have endogenous defences against oxidants. However, problems occur when production of ROS exceeds elimination, either due to overproduction during trauma and detoxification, or due to damage to the natural antioxidant system. Such an imbalance in the amount of ROS produced, and ROS that is eliminated, is called oxidative stress. This can cause cellular damage to lipids, proteins and DNA, and subsequent death.

### 2.1 Sources of ROS

Sources of environmental ROS are ultraviolet light ionising radiation, (air) pollutants, smoking, ingestion of alcohol or other toxins that produce an excess of ROS during detoxification.

In the body the most important sources of ROS are enzymatic driven redox reaction involving oxygen. Oxygen, being the most abundant molecule in biological systems, exists as a biradical and is therefore extremely reactive with other radicals. Oxygen itself is often the source of such radicals as partially reduced species are generated through normal metabolic processes, and some of these reactive species can escape<sup>3</sup>. Continuous production of small amount of free radicals and ROS is therefore a basic feature of aerobic life. Within certain boundaries, the generation of ROS is even essential to maintain homeostasis. For example, ROS generation by phagocytic cells constitutes an essential host defence mechanism necessary to combat infection. Likewise, cytosolic ROS produced in response to stimulation by growth factors are involved in regulating the proliferative response. Under certain situation of metabolic stress, even mitochondrial-derived oxidants seem to function as signalling molecules. However a more extreme rise in intracellular oxidant levels has two

potentially important effects: damage to various cell components and triggering of the activation of specific signalling pathways <sup>4</sup>.

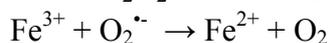
The most abundant source of  $O_2^{\cdot-}$ , being the major factor in oxygen toxicity <sup>5</sup>, in the body is the mitochondrial electron transport chain, where the synthesis of adenosine triphosphate (ATP) is coupled to the reduction of  $O_2$ .  $O_2^{\cdot-}$  can be formed at Complex I, by the donation of an electron to oxygen from nicotinamide adenine dinucleotide (NADH) dehydrogenase.  $O_2^{\cdot-}$  and/or  $H_2O_2$  may also be formed following the auto-oxidation of reduced ubiquinone at Complex II (succinate-coenzyme Q) and/or Complex III (coenzymeQH<sub>2</sub>-cytochrome c reductases) sites. Making the generation of ROS predominantly a function of metabolic rate. Another important metabolic way where ROS are produced is by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase of the antibacterial function on phagocytic cells. Production of  $O_2^{\cdot-}$  is an important part of the bacterial killing mechanism of neutrophils or polymorphonuclear leukocytes (PMNs). In this process, called phagocytosis invading microorganisms are enclosed in phagosomes. During the respiratory burst the PMNs become activated,  $O_2$  consumption increases and a membrane-associated NADPH oxidase reduces oxygen to superoxide anion ( $O_2^{\cdot-}$ ) <sup>6</sup>, which subsequently dismutates to form  $H_2O_2$  <sup>7</sup>. However the expanding family of NADPH oxidases include as well the nitric oxide synthase (NOS) family, illustrating the apparent purposeful and deliberate use of oxidant generation in normal cellular signalling and homeostasis.

## 2.2 Reactivity

Among the ROS, the  $HO^{\cdot}$  is the most reactive and can attack almost every molecule in the body. Because it is so reactive, it does not diffuse very far from its point of synthesis and its half-life therefore is diffusion limited at about a nanosecond. It is produced via the Haber-Weiss reaction:



in the following manner:



As described previously  $O_2^{\cdot-}$  is produced from the electron transport chain and during oxidative burst by neutrophils and phagocytes using NADPH oxidase. It is not lipid soluble and therefore cannot diffuse very far from its point of synthesis, it has a half life of about a microsecond.

$H_2O_2$  is a mild oxidising agent, is relatively stable, and easily moves through cell membranes and affects sites far from its origin. It is generated from superoxide dismutase (an enzyme that eliminates  $O_2^{\cdot-}$ ), when ascorbic acid reduces  $O_2^{\cdot-}$  and it is produced in large amounts

during beta-oxidation of long-chain fatty acids. However, it is believed that  $\text{H}_2\text{O}_2$  and  $\text{O}_2^{\cdot-}$  produce their most potent effects by forming the hydroxyl radical ( $\text{OH}^{\cdot}$ ) via the Haber-Weiss reaction (see previous).

### 3. Oxidative stress

When cellular production of ROS overwhelms its antioxidant capacity, damage to molecules (and accumulation thereof) and disruption in cellular homeostasis may ensue (Figure 1). Free radicals, especially  $\text{OH}^\bullet$ , which as described is a highly reactive species interacting with almost anything in its immediate vicinity, can react with macromolecules such as proteins and carbohydrates, lipids (particularly polyunsaturated fatty acids) and DNA.

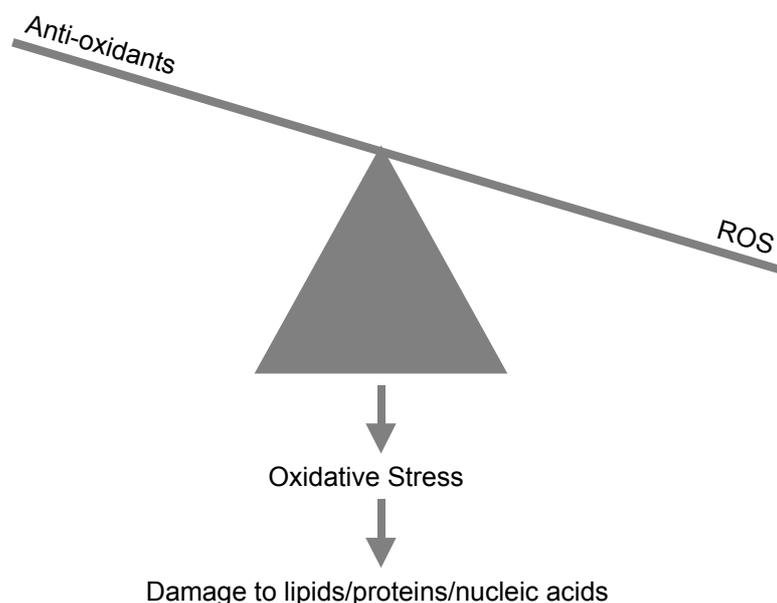


Figure 1. Schematic view of oxidative stress resulting in damage to macromolecules.

#### 3.1 Protein

Proteins are vulnerable to oxidation by ROS at different levels. Protein oxidation results in an increase of protein carbonyl levels and glutamine synthetase activity<sup>8</sup>. The protein carbonyls can be assayed by the stable hydrazone derivatives formed with 2,4-dinitrophenyl hydrazine<sup>9</sup>. When ROS attacks proteins formed carbonyls can react with nitrogen species and then with tyrosine to form nitrotyrosine<sup>10,11,12</sup>. In a similar way bromotyrosine can be formed<sup>13,14</sup>. Oxidation of proteins and carbohydrates may result in fragmentation at mainly proline and histidine residues and cross-linking, both with subsequent loss of function. Metal binding sites of proteins make them more susceptible to damage through pro-oxidant effects of metals. Reactive sulfhydryl groups such as cysteine and methionine residues undergo reversible modification. Protein repair systems do not appear to exist since it is apparently more efficient to either prevent oxidation or to simply destroy the modified species.

## 3.2 Lipids

Peroxidation of lipid in membranes severely impairs membrane function. Lipid peroxides produce an irreversible impairment of membrane fluidity and elasticity, which can cause rupture of the cell. Lipid oxidation is self-propagating in cellular membranes. Products of lipid peroxidation are easily measured and an increased level of lipid peroxidation is the evidence most frequently cited in support of the involvement of oxidative stress in tissues. Malondialdehyde (MDA) is produced during lipid peroxidation<sup>8</sup> and can be measured using GC-MS<sup>15</sup>. It is a known mutagen, reacting with proteins and amino acids. A peroxidation sequence in a membrane or polyunsaturated fatty acid (PUFA) is initiated by the attack of any free radical with sufficient reactivity to remove a hydrogen atom from a methylene group of an unsaturated fatty acid. This carbon-centred lipid radical then quickly reacts with molecular oxygen to produce a hydroperoxyl radical. The peroxy radical can then propagate the oxidising chain reaction by taking electrons from other susceptible PUFA, forming another lipid free radical and a molecule of lipid hydroperoxide.

ROS react with lipids to liberate ethane<sup>16</sup> and isoprostanes<sup>17,18</sup>. Isoprostanes are considered to be a marker of oxidative stress and many disease states<sup>19</sup>. They may in fact mediate the effects of free radicals and reactive oxygen species<sup>20</sup> and have the ability to affect signalling pathways including those that regulate the apoptotic form of cell death<sup>3</sup>. For example, the non-enzymatic peroxidation of arachidonic acid (normally enzymatically metabolised by cyclo-oxygenase (COX) to form a wide variety of prostanoids (prostaglandins and thromboxanes)) by free radicals and reactive oxygen species produces isoprostanes.

Overall lipid peroxidation is the most common index of oxidative stress and can be estimated by the determination of non-specific thiobarbituric acid-reactive substances (TBARS) as described by Rhoden *et al.*<sup>21</sup>, as well commercially available (Oxitek, Brussels, Belgium). However it has been suggested that this assay might overestimate lipid peroxidation when compared with a more specific gas chromatography-mass spectrometry (GC-MS assay)<sup>8</sup>.

## 3.3 DNA

In humans, oxidative damage to DNA has been estimated at 10000 hits per cell per day. ROS cause modifications in bases, which leads to genetic defects if the modifications are not repaired. The DNA base guanine is particularly sensitive to oxidation making the detection of 8-hydroxy-deoxyguanosine a reasonable biomarker for oxidative injury<sup>22</sup>. Besides this also thymidine glycol is used to measure DNA damage due to ROS degradation of bases. ROS can cause cross-linking of DNA with DNA or protein. This occurs less often but is more difficult to repair.

## 4. Antioxidant mechanisms

As a basic defence mechanism towards ROS, removal of O<sub>2</sub>, scavenging of ROS and precursors, inhibition of ROS, chelation of metal ions, and upregulation of endogenous antioxidant defences occurs.

The burden of ROS production is largely counteracted by a complex antioxidant defence system. An antioxidant is defined as any substance that, when present at low concentrations compared with those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate<sup>23</sup>.

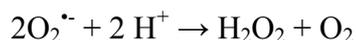
Classification of antioxidants can be based on their way of action, enzymatic and non-enzymatic scavengers, or for example on their site of action, in intra-cellular and extra-cellular. Intra-cellular scavengers include glutathione peroxidase, glutathione, catalase, superoxide dismutase. Extra-cellular antioxidants include vitamin E, vitamin C, carotenoids, uric acid, plasma proteins such as metal chelators and albumin<sup>24</sup>.

It seems likely that especially in diseased persons, protective antioxidant mechanisms are reduced leading to an imbalance between free radical production and removal, resulting in accumulation of end products of free radical action and slow progressive tissue damage<sup>24</sup>.

### 4.1 Antioxidant enzymes

#### *Superoxide dismutase*

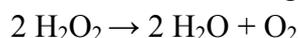
Superoxide dismutase (SOD) converts superoxide to hydrogen peroxide in the following way:



Two different forms of SOD exist, a CuZn SOD which is found in cytoplasm and extracellular fluid, and MnSOD found in mitochondria.

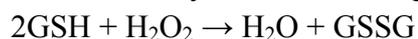
#### *Catalase*

Catalase is a heme-containing enzyme located in the peroxisomes, which detoxifies H<sub>2</sub>O<sub>2</sub>:



#### *Glutathione peroxidase*

Glutathione peroxidase is a selenium (Se) containing enzyme found in the cytosol and mitochondria. It catalyses the following reaction:



#### *Thioredoxin and glutaredoxin systems*

Thioredoxin (Trx) and glutaredoxin (Grx) catalyse thiol-disulfide exchange reactions required for many functions including general regulation of protein function by thiol redox control. Trx is reduced to the dithiol form by NADPH and thioredoxin reductase (together

called the thioredoxin system). Grx in contrast is reduced by the ubiquitous glutathione (GSH) and GSSG in turn is reduced by NADPH and glutathione reductase (together called the glutaredoxin system). The thioredoxin and glutaredoxin systems induced by a variety of stress conditions including viral infection, ischemic insult, exposure to UV light, X-ray irradiation, and hydrogen peroxide exposure<sup>25</sup>. *In vitro* and *in vivo* studies have demonstrated that these systems have a protective effect against ROS-induced cellular damage<sup>26,27</sup>.

#### *Hemeoxygenase*

The stress-responsive and antioxidant enzyme heme oxygenase (HO-1) is known to be induced by various oxidative agents. For example, HO-1 mRNA showed the most marked response to organic extracts of diesel exhaust particles (OE-DEP) in rat heart microvessel endothelial (RHMVE) cells<sup>28</sup>.

#### *Glutamine synthetase*

Glutamine synthetase is a key enzyme in cellular nitrogen regulation facilitating the uptake of excitatory neurotransmitter glutamate and is among the enzymes that are rapidly induced by stress hormone glucocorticoids. A decreased uptake of glutamate due to decreased glutamine synthetase activity could result in neurotoxic effects of abnormally prolonged N-methyl-D-aspartate receptor activation and generation of oxidants<sup>29</sup>. Therefore, an increase indicates a stress response.

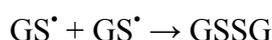
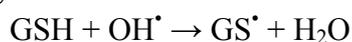
## **4.2 Antioxidant proteins**

Among antioxidant proteins we can distinguish iron and copper sequestering proteins (such as ceruloplasmin, albumin, transferrin) as well proteins that bind free heme (such as haptoglobin, hemopexin). A special group form the metallothioneins (MTs) which are ubiquitous low molecular weight proteins of extremely high metal and sulphur content. They are thought to play a role in the protection from a variety of stress conditions, and have been recognised as potentially having antioxidant activity.

## **4.3 Low molecular weight antioxidants**

#### *Glutathione*

Glutathione (GSH) is abundant in cytoplasm, nuclei, and mitochondria and is the major water-soluble antioxidant in these cell compartments (it is present in millimolar concentrations within cells). The sulphur atom easily loses a single electron and reacts directly with OH<sup>•</sup>



Glutathione is the major redox buffer of several eukaryotic cell types and an important factor in the cellular defence against oxidative stress. This is due to its own antioxidant capacity and

because this thiol is involved in the recycling of other antioxidants. Glutathione homeostasis is regulated by several enzymes, such as glutathione reductase (GR), glutathione peroxidase (GPX), and glutathione transferase (GST). It has been suggested that the capacity of the GSH redox cycle, rather than the intracellular levels of reduced GSH, determines the resistance to oxidative stress at least in some cell types. The ratio between reduced GSH and total GSH is a good indicator of oxidative stress (reduction with increased oxidative stress). As well down regulation of GPX/GR ratio with increased oxidative stress <sup>30</sup>.

#### *Tocopherol*

Tocopherol (vitamin E) is lipid soluble and resides almost exclusively on or at the surface of cell membranes. Vitamin E has a chromanol ring which is a hydroxylated aromatic ring, and several isoprene side chains.

#### *Ascorbic acid*

Ascorbic acid (vitamin C) is the most versatile and effective water-soluble antioxidant. It efficiently scavenges  $\text{OH}^\bullet$ ,  $\text{ROO}^\bullet$ , singlet oxygen and reactive peroxides. It prevents lipid peroxidation by scavenging peroxy radicals in the aqueous phase and regenerating vitamin E. It is known that high levels of ascorbic acid in neutrophils protect host tissue during oxidative burst. It is recycled to reduced state by GSH and NADPH.

Other low molecular weights antioxidants are carotenoids, uric acid, lipoic acid and melatonin.

## 5. Signal transduction

Besides ROS being part of an oxidative stress situation, as mentioned briefly in the introduction, recent studies have also implicated ROS that are generated by specialised plasma membrane oxidase in *normal* physiological signalling by growth factors and cytokines<sup>31</sup>.

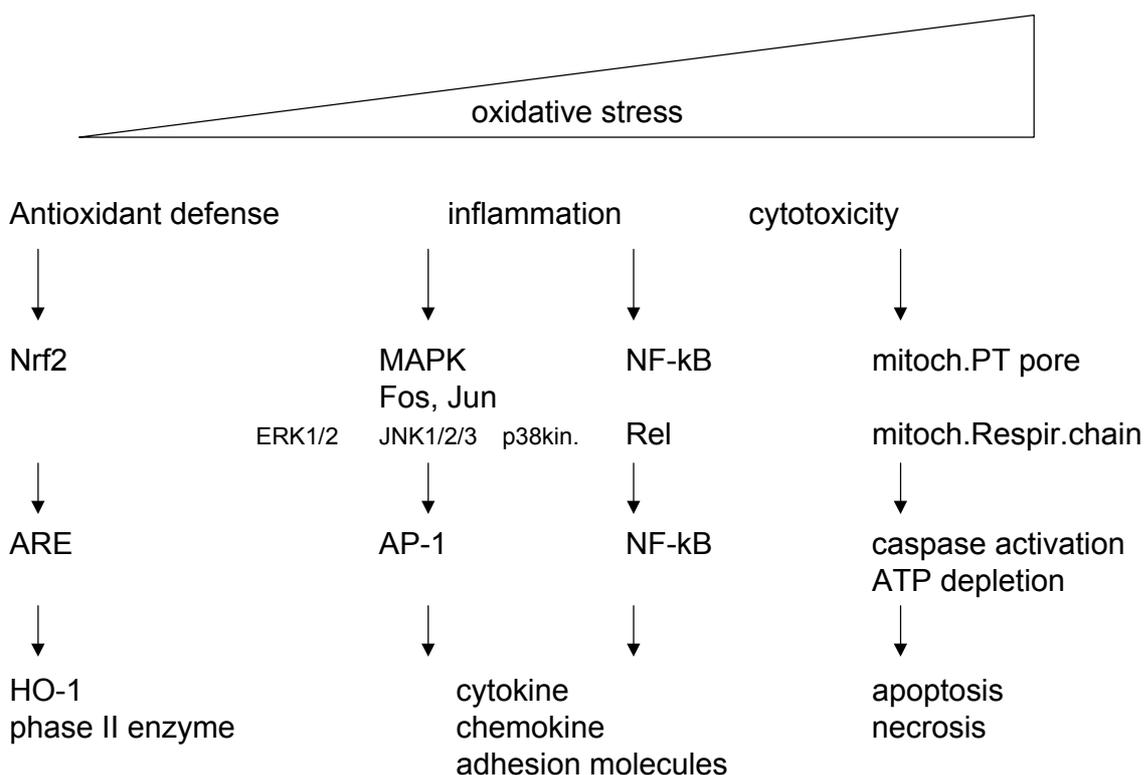


Figure 2. Oxidative stress model in response to PM (adapted from Li et al.<sup>1</sup>). Different levels of oxidative stress can be distinguished. At a lower level of oxidative stress antioxidant enzymes are induced to restore cellular redox homeostasis. At an intermediate level an inflammation response is induced, via activation of MAPK and NFκB pathways. And at the highest level cytotoxicity results in cellular apoptosis or necrosis.

ROS are able to act directly on signal transduction pathways, or indirectly through the generation of bioactive mediators. ROS modulate quite a number of, so-called redox sensitive transcription factors, such as activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB) (Figure 2). Whereas ROS are also able to affect activity of calcium signalling, antioxidant enzymes, ion transporters, various cell growth related genes and, various kinases (c-jun N terminal kinase (JNK), extracellular regulated kinase (ERK) and the mitogen activated protein kinase (MAP kinase) family)<sup>32,33</sup>. It seems that also controlling the thiol status of a cell is critical in altering the signalling pathway.

## 6. Situation in the lung

### 6.1 Oxidative stress

As stated above in its simplest form, oxidative stress is a potentially harmful process, that occurs when there is an excess of oxidants, a decrease in antioxidant defences, or a combination of these events (Figure 1). As a consequence, free radicals attack and oxidise other cell components such as lipids, proteins, and nucleic acids. Signals can consequently lead to the activation of transcription factors and increased expression of pro-inflammatory chemokines and cytokines (such as  $\text{TNF}\alpha$ , IL-6 etc.). These signalling molecules lead to the up regulation of adhesion molecules. Consequently immune cells (neutrophils, eosinophils, and macrophages) move into the lung tissue. NADPH oxidase on these cells is activated, and using  $\text{O}_2$ , produces  $\text{O}_2^{\cdot-}$ . Removal of this reactive oxygen species by SOD results in generation of  $\text{H}_2\text{O}_2$ , which can easily pass across cell membranes. It might activate intracellular signalling pathways or generate other ROS ( $\text{HO}^{\cdot}$  in the presence of metals,  $\text{HOCl}$  by myeloperoxidase which is abundantly present in neutrophils). This highly organised series of events can therefore lead to a second wave of oxidative stress in the lung (6-18 hours after the initial exposure). In the absence of any invading organisms to kill, the generated and released free radicals attack local tissue components and cause cell injury.

Reactive oxygen intermediates are thought to be primary mediators of macrophage-induced tissue injury, and they have been implicated in the pathogenesis of xenobiotic induced lung toxicity<sup>34</sup>. Thus following inhalation of irritants, pulmonary levels of reactive oxygen intermediates increase in the lung<sup>34,35</sup>. Moreover, antioxidants have been shown to avoid tissue injury in several of these models.

### 6.2 Antioxidant

The lung has a well-developed antioxidant system. First of all the conducting airways are protected by an epithelial lining fluid (ELF) containing low molecular weight antioxidants such as uric acid, ascorbic acid, and GSH, as well as antioxidant proteins such as albumin and lactoferrin. Ascorbic acid is known to be positively related to pulmonary function.

Tocopherols are believed to be the first line of defence against membrane oxidation.

The lung cells by themselves possess relatively high levels of antioxidants such as SOD, catalase, and GSH to prevent or limit oxidative damage<sup>36,37</sup>. However, during states of increased oxidative stress antioxidants become depleted, exacerbating tissue injury<sup>34</sup>.

For the lung SOD is generally considered one of the first lines of antioxidant defence<sup>38</sup>. As described previously this enzyme converts  $\text{O}_2^{\cdot-}$  into  $\text{H}_2\text{O}_2$ , which is then removed by catalase. Antioxidant enzymes are not uniformly distributed throughout the cells of the lung, which implies that some cell types may be relatively unprotected<sup>39</sup>. MnSOD protein is primarily expressed in the mitochondria of type II alveolar cells, with lung fibroblasts containing very

little and type I alveolar cells and endothelial cells containing no detectable immunoreactive protein.

### *GSH*

Special attention deserves GSH, being one of the key components of the well-developed antioxidant system of the lung<sup>40</sup>. GSH, being a vital intra- and extracellular protective antioxidant, has been shown to be critical in protecting airspace epithelium from oxidative/free radical mediated injury and inflammation<sup>41</sup>. It protects cells from the toxic effects of oxidants by maintaining the reduced state of the sulfhydryl groups of a number of sulfhydryl-dependent enzymes<sup>42</sup>. Induction of oxidative stress is evident by decreased levels of the antioxidant GSH in the lungs<sup>43</sup>, and epithelial cells *in vitro*. Acute inhalation of oxidising agents such as ozone or NO<sub>2</sub> depletes the lungs of GSH<sup>44</sup>. Depletion of GSH, affecting the GSH/GSSG ratio, influences a variety of cellular signalling processes, such as activation of the transcription factors AP-1 and nuclear factor kappa B (NF-κB) (Figure 2). It as well can increase permeability of lung epithelium, which can lead to passage of particles into the interstitium<sup>41</sup>. Alterations in the alveolar and lung GSH metabolism are widely recognised as a central feature of many inflammatory lung diseases.

The intracellular GSH redox homeostasis is strictly regulated to govern cell metabolism and protect cells against oxidative stress<sup>45</sup>. The synthesis of glutathione requires two enzymes and three amino acids, glycine, cysteine and glutamic acid (with cysteine being the rate-limiting substrate). The tripeptide GSH is formed by the consecutive actions of γ-GCS and glutathione synthetase. Gamma-glutamylcysteine synthetase (γ-GCS) is the rate-limiting enzyme in GSH synthesis. γ-GCS consists of a catalytic heavy subunit (γ-GCS-HS) and a regulatory light subunit (γ-GCS-LS). The promoter regions of both corresponding genes contain putative AP-1 and antioxidant response element (ARE) response elements necessary for expression in response to diverse stimuli, such as oxidants, phenolic antioxidants, inflammatory, and anti-inflammatory agents in lung cells.

## **6.3 Signal transduction**

The development of an oxidant/antioxidant imbalance in lung inflammation may activate redox-sensitive transcription factors such as NF-κB, and AP-1<sup>46,47</sup>. Transcription factors, are DNA-binding proteins, that interact with specific sequence motifs in the promoter region of the gene, controlling the expression of these. In particular NF-κB, a multisubunit transcription factor, plays a central role in the regulation of the expression of numerous genes involved in the inflammatory and immune response (resulting in upregulation of cytokines and chemokines)<sup>48</sup> as well as protective antioxidant genes, such as γ-GCS. The critical balance between the induction of proinflammatory mediators and antioxidant genes in response to oxidative stress at the site of inflammation is not known<sup>49</sup>. Cellular oxidative processes seem also to activate stress kinases (JNK, MAPK, p38).

Exogenous and endogenous antioxidants are effective in blocking activation of NF- $\kappa$ B and preventing the consequences of proinflammatory gene expression. Phase II enzymes either directly or indirectly play a major *in vivo* role in minimising oxidative stress by scavenging peroxides, peroxide breakdown products and dicarbonyls and in regeneration of lipid peroxidation chain-breaker, vitamin E<sup>50</sup>.

As an antioxidant defence, induction of these genes, via mainly the ARE pathway, is a first step trying to restore cellular redox homeostasis (Figure 2). However during a prolonged situation of oxidative stress activation of MAPK and NF- $\kappa$ B cascade induces proinflammatory responses. Expression of many proinflammatory mediators such as cytokines (IL-1b, TNF $\alpha$ , IL-6), chemokines (IL-8 and MIP-2), adhesion molecules (VCMAM-1, ICAM-1, and E-selectin), and enzymes (iNOS) are regulated by NF- $\kappa$ B<sup>51,52</sup>. Aside from NF- $\kappa$ B, other transcription factors such as activator protein-1, interferon regulatory factors, and signal transducers and activators of transcription are also involved in the induction of proinflammatory cytokines<sup>53</sup>. At the lowest levels of oxidative stress cytoprotective pathways are induced resulting in expression of antioxidant enzymes, such HO-1 and catalase<sup>54</sup>. Induction of HO-1 expression is dependent on the ARE in the promoter of that gene, which is transcriptionally activated by a basic leucine zipper transcription factor, NF-E2-related factor-2 (Nrf2). In a next step (second tier of oxidative stress responses) the p38MAPK and Jun kinase cascades are activated resulting in activation of NF- $\kappa$ B and AP-1. Whereas under extreme high levels of oxidative stress the final tier of response, mediated by mitochondrial perturbation, leads to cytotoxic effects (see Figure 2).

## 7. Ozone and PM exposure

Since ozone is a well-known and long-time studied oxidative air-pollution it is used within S/630111/01/AB as a model compound for generating oxidative stress by exposure.

### 7.1 Ozone exposure

Inhalation of ozone primarily affects the terminal bronchioles and the alveoli. The bronchiolar ciliated cells and the epithelial alveolar type I cells are damaged, and are later replaced with proliferating Clara cells in the airways and epithelial type II cells in the alveoli, respectively.

It is believed that ozone operates via two main mechanisms of action. First by the oxidation of polyunsaturated fatty acids under the formation of acid peroxides and dialdehydes such as malonaldehyde<sup>55</sup>. Second by oxidation of sulfhydryl groups and amino acids of proteins. Exposure to ozone is associated with increase in cellular and biochemical markers of inflammation in bronchoalveolar lavage fluid (BALF) and induced sputum<sup>56,57</sup>. Ozone exposure causes an airway influx and activation of neutrophils (PMNs)<sup>56</sup>, which induces a respiratory burst resulting in overproduction of ROS and H<sub>2</sub>O<sub>2</sub><sup>58</sup>. ROS generation is associated with lipoperoxidation of airway cell membranes and subsequent release of a number of molecules resulting from the cleavage of arachidonic acid, including malondialdehyde (MDA)<sup>59</sup>, 8-isoprostane<sup>60</sup>, and the PMN chemotactic mediator leukotrine B-4 (LTB4)<sup>61</sup>.

In exhaled breath condensate (EBC) a sudden increase in 8-isoprostane levels, as well as H<sub>2</sub>O<sub>2</sub>, and a late increase (after 18 h) in LTB-4 and TBARS levels is observed<sup>62</sup>.

Biochemical studies have demonstrated that ozone exposure stimulates the induction of the glutathione peroxidase system. The induction of this system is thought to be a protective mechanism. Another activated protective mechanism is the induction of SOD, following exposure of animals to ozone<sup>63,64</sup>. Whereas Fakhrzadeh *et al.* have shown that SOD overexpressing mice are resistant to ozone-induced tissue injury and increases in NO and TNF $\alpha$ , it is believed that macrophages and inflammatory mediators contribute to ozone-induced lung inflammation and toxicity<sup>65</sup>. Since ozone increases NO production by alveolar macrophages and alveolar type II epithelial cells it is believed that NO has an adverse role in ozone-induced lung injury<sup>66</sup>.

### 7.2 PM exposure

Currently, three theories of how particles induce oxidative stress have been suggested. Firstly, particles contain several soluble transition metals on the surface which induce generation of reactive oxygen species (ROS) in biological systems<sup>67,68,69</sup>. The second

mechanism involves the organic fraction of particles, which consists of several redox-active quinones, existing or generated during metabolism of several polycyclic aromatic hydrocarbons (PAH). Quinones produce ROS during their metabolism by redox cycling, unless they are catalysed by the phase II enzyme NAD(P)H:quinone oxidoreductase-1 (NQO1). The third mechanism involves reactions by alveolar macrophages (AM) and neutrophils. These are phagocytes known to ingest and remove inhaled particles from the lungs<sup>70</sup>. This leads to activation of these cells causing release of several cytokines as well as ROS through a 'respiratory burst'<sup>71</sup>. It is unclear which components of ambient particles are responsible for phagocyte activation.

Long term exposure to high particle levels increase risk of cancer, respiratory diseases and arteriosclerosis, whereas short-term exposure-peaks cause exacerbation of bronchitis, asthma and other respiratory diseases as well as changes in heart rate variability<sup>72,73,74</sup>. Low concentrations exposure assessment and establishing relationships with biological effects may be difficult. By means of monitors of individual exposure to air pollutants and of biomarkers of internal dose, biologically effective dose and early biological effect as well as markers of individual susceptibility, such relationships may be documented.

Transition metals such as iron and copper being part of PM are capable of redox cycling and can induce Fenton reaction, resulting in the production of reactive oxygen and nitrogen species<sup>75</sup>. It has been shown that equal masses of PM can induce disparate lung injuries, suggesting that particle components may be relevant in assessing health effects after their exposure. In the case of the Utah Valley study, specifically metals can participate in the biologic effects of PM collected.

In a recent experiment rats were exposed to PM and subsequently gene expression in the lung tissue was analysed. Strong induction of the following genes was observed, metallothionein, heme oxygenase, thioredoxin reductase, glutathione reductase and SOD as well as phase II enzymes such as P450.

As shown in Figure 2, at the lowest levels of oxidative stress cytoprotective pathways are induced resulting in expression of antioxidant enzymes, such HO-1 and catalase and phase II enzymes<sup>54</sup>. Induction of HO-1 expression is dependent on the ARE in the promoter of that gene, which is transcriptionally activated by a basic leucine zipper transcription factor, Nrf2. PAH, organic component carried on the particle surface, can induce oxidative stress indirectly, through biotransformation by cytochrome P450 and dihydrodiol dehydrogenase to generate redox active quinones that act as catalysts for free radical production.

## 8. Conclusion biomarkers for oxidative stress

It is clear from the previous that oxidative stress is a very complicated process, which can be described as an imbalance between ROS and antioxidants resulting in damage to lipids, proteins and nucleic acids. Monitoring this process of oxidative stress is not easy since ROS are very reactive particles and therefore difficult to measure. Therefore measurements of products, macromolecules damaged by reactive oxygen species, is the most common technique to indirectly measure oxidative stress. These products, often called footprints, are not specific for individual reactive oxygen species. Many oxidative footprints are thought to be the result of nonenzymatic reactions between reactive oxygen species and organic molecules, such as proteins, lipids, or DNA <sup>76</sup>.

Another way to monitor the situation of oxidative stress is measurement of the primary defence against ROS, the endogenous antioxidants. Changes in these antioxidants levels and its balance with ROS might give us information about the state of oxidative stress (Figure 1). It might well be that certain risk groups in the population are more susceptible for air pollution due to an off balance between ROS and antioxidants.

Based on the previous given overview on oxidative stress, consisting of ROS, oxidation products and the response to it, the following list of biomarkers is created (Table 1). This list of biomarkers is valuable to investigate oxidative stress as a mechanism in health effects due to air pollution.

*Table 1. Biomarkers of oxidative stress*

	biomarker	reference
<i>Oxidation products</i>		
Protein	Carbonyl content	9
Lipids	MDA	59
	Plasma isoprostane, 8-isoprostane	19, 60
	Overall lipid peroxidation, TBARS	21
DNA	8-hydroxy-2'-deoxyguanosin	
<i>Antioxidant</i>		
Enzymes	SOD	77
	Catalase	
	Hemeoxygenase	77
	Metallothioneins	77
	Thioredoxin system	77
	Glutaredoxin system	77
LMW antioxidants	GSH	

It has been shown that inhalation exposure to concentrated ambient particles (CAPs) increases two-fold the steady-state concentration of ROS in the rat lung and heart <sup>68</sup>. These

CAPs dependent increases in ROS are sufficient to cause accumulation of oxidised lipids and proteins and about a two-fold increase in the levels of TBARS and protein carbonyls was measured <sup>21</sup>. Therefore both oxidation products are valuable markers for oxidative stress in the lung. The oxidation product of the DNA base guanine, 8-hydroxy-deoxyguanosine, is a reasonable biomarker for oxidative injury <sup>22</sup>.

In the antioxidant response to oxidative stress, SOD and catalase are two important enzymes since they directly catalyse the reaction of the ROS,  $O_2^{\cdot-}$  and  $H_2O_2$ . The response of these and other enzymes is very well followed by expression of their mRNA (Southern blotting, gene expression arrays, real time PCR). A recent gene expression study performed by our group with spontaneous hypertensive rats exposed to particulate matter showed increases of SOD, glutathion reductase, heme oxygenase, metallothioneins, and thioredoxin reductase <sup>77</sup>.

In respect to the LMW antioxidants the intra- and extracellular protective antioxidant GSH is probably the most important antioxidant in the lung <sup>40</sup>. It protects cells from the toxic effects of oxidants by maintaining the reduced state of the sulfhydryl groups of a number of sulfhydryl-dependent enzymes <sup>42</sup>. Changes in the levels of GSH or GSH/(total glutathion) ratio, therefore is a very important biomarker of oxidative stress.

## References

- (1) Li, N.; Hao, M.; Phalen, R. F.; Hinds, W. C.; Nel, A. E. Particulate Air Pollutants and Asthma. A Paradigm for the Role of Oxidative Stress in PM-Induced Adverse Health Effects. *Clin. Immunol.* 2003, 109, 250-265.
- (2) Kelly, F. J. Oxidative Stress: Its Role in Air Pollution and Adverse Health Effects. *Occup. Environ. Med.* 2003, 60, 612-616.
- (3) Kehrer, J. P. The Haber-Weiss Reaction and Mechanisms of Toxicity. *Toxicology* 2000, 149, 43-50.
- (4) Finkel, T.; Holbrook, N. J. Oxidants, Oxidative Stress and the Biology of Ageing. *Nature* 2000, 408, 239-247.
- (5) Fridovich, I. Superoxide Dismutases. *Adv. Enzymol. Relat Areas Mol. Biol.* 1974, 41, 35-97.
- (6) Babior, B. M.; Kipnes, R. S.; Curnutte, J. T. Biological Defense Mechanisms. The Production by Leukocytes of Superoxide, a Potential Bactericidal Agent. *J. Clin. Invest* 1973, 52, 741-744.
- (7) Weening, R. S.; Wever, R.; Roos, D. Quantitative Aspects of the Production of Superoxide Radicals by Phagocytizing Human Granulocytes. *J. Lab Clin. Med.* 1975, 85, 245-252.
- (8) Liu, J.; Yeo, H. C.; Overvik-Douki, E.; Hagen, T.; Doniger, S. J.; Chyu, D. W.; Brooks, G. A.; Ames, B. N.; Chu, D. W. Chronically and Acutely Exercised Rats: Biomarkers of Oxidative Stress and Endogenous Antioxidants. *J. Appl. Physiol* 2000, 89, 21-28.
- (9) Levine, R. L.; Williams, J. A.; Stadtman, E. R.; Shacter, E. Carbonyl Assays for Determination of Oxidatively Modified Proteins. *Methods Enzymol.* 1994, 233, 346-357.
- (10) Hanazawa, T.; Kharitonov, S. A.; Barnes, P. J. Increased Nitrotyrosine in Exhaled Breath Condensate of Patients With Asthma. *Am. J. Respir. Crit Care Med.* 2000, 162, 1273-1276.
- (11) Kaminsky, D. A.; Mitchell, J.; Carroll, N.; James, A.; Sulttanakis, R.; Janssen, Y. Nitrotyrosine Formation in the Airways and Lung Parenchyma of Patients With Asthma. *J. Allergy Clin. Immunol.* 1999, 104, 747-754.
- (12) Paredi, P.; Kharitonov, S. A.; Hanazawa, T.; Barnes, P. J. Local Vasodilator Response to Mobile Phones. *Laryngoscope* 2001, 111, 159-162.
- (13) Heinecke, J. W. Eosinophil-Dependent Bromination in the Pathogenesis of Asthma. *J. Clin. Invest* 2000, 105, 1331-1332.

- (14) Wu, W.; Samoszuk, M. K.; Comhair, S. A.; Thomassen, M. J.; Farver, C. F.; Dweik, R. A.; Kavuru, M. S.; Erzurum, S. C.; Hazen, S. L. Eosinophils Generate Brominating Oxidants in Allergen-Induced Asthma. *J. Clin. Invest* 2000, 105, 1455-1463.
- (15) Liu, J.; Yeo, H. C.; Doniger, S. J.; Ames, B. N. Assay of Aldehydes From Lipid Peroxidation: Gas Chromatography-Mass Spectrometry Compared to Thiobarbituric Acid. *Anal. Biochem.* 1997, 245, 161-166.
- (16) Paredi, P.; Kharitonov, S. A.; Barnes, P. J. Elevation of Exhaled Ethane Concentration in Asthma. *Am. J. Respir. Crit Care Med.* 2000, 162, 1450-1454.
- (17) Dworski, R.; Roberts, L. J.; Murray, J. J.; Morrow, J. D.; Hartert, T. V.; Sheller, J. R. Assessment of Oxidant Stress in Allergic Asthma by Measurement of the Major Urinary Metabolite of F2-Isoprostane, 15-F2t-IsoP (8-Iso-PGF2alpha). *Clin. Exp. Allergy* 2001, 31, 387-390.
- (18) Montuschi, P.; Corradi, M.; Ciabattini, G.; Nightingale, J.; Kharitonov, S. A.; Barnes, P. J. Increased 8-Isoprostane, a Marker of Oxidative Stress, in Exhaled Condensate of Asthma Patients. *Am. J. Respir. Crit Care Med.* 1999, 160, 216-220.
- (19) Devlin, R. B. Identification of Subpopulations That Are Sensitive to Ozone Exposure: Use of End Points Currently Available and Potential Use of Laboratory- Based End Points Under Development. *Environ. Health Perspect.* 1993, 101 Suppl 4, 225-230.
- (20) Janssen, L. J. Isoprostanes: Generation, Pharmacology, and Roles in Free-Radical-Mediated Effects in the Lung. *Pulm. Pharmacol. Ther.* 2000, 13, 149-155.
- (21) Rhoden, C. R.; Lawrence, J.; Godleski, J. J.; Gonzalez-Flecha, B. N-Acetylcysteine Prevents Lung Inflammation After Short-Term Inhalation Exposure to Concentrated Ambient Particles. *Toxicol. Sci.* 2004, 79, 296-303.
- (22) Tsuboi, H.; Kouda, K.; Takeuchi, H.; Takigawa, M.; Masamoto, Y.; Takeuchi, M.; Ochi, H. 8-Hydroxydeoxyguanosine in Urine As an Index of Oxidative Damage to DNA in the Evaluation of Atopic Dermatitis. *Br. J. Dermatol.* 1998, 138, 1033-1035.
- (23) Halliwell, B. Reactive Oxygen Species in Living Systems: Source, Biochemistry, and Role in Human Disease. *Am. J. Med.* 1991, 91, 14S-22S.
- (24) Bunker, V. W. Free Radicals, Antioxidants and Ageing. *Med. Lab Sci.* 1992, 49, 299-312.
- (25) Nakamura, H.; Nakamura, K.; Yodoi, J. Redox Regulation of Cellular Activation. *Annu. Rev. Immunol.* 1997, 15, 351-369.
- (26) Fukuse, T.; Hirata, T.; Yokomise, H.; Hasegawa, S.; Inui, K.; Mitsui, A.; Hirakawa, T.; Hitomi, S.; Yodoi, J.; Wada, H. Attenuation of Ischaemia Reperfusion Injury by Human Thioredoxin. *Thorax* 1995, 50, 387-391.
- (27) Sasada, T.; Iwata, S.; Sato, N.; Kitaoka, Y.; Hirota, K.; Nakamura, K.; Nishiyama, A.; Taniguchi, Y.; Takabayashi, A.; Yodoi, J. Redox Control of Resistance to Cis-Diamminedichloroplatinum (II) (CDDP): Protective Effect of Human Thioredoxin Against CDDP-Induced Cytotoxicity. *J. Clin. Invest* 1996, 97, 2268-2276.

- (28) Hirano, S.; Furuyama, A.; Koike, E.; Kobayashi, T. Oxidative-Stress Potency of Organic Extracts of Diesel Exhaust and Urban Fine Particles in Rat Heart Microvessel Endothelial Cells. *Toxicology* 2003, 187, 161-170.
- (29) Smith, C. D.; Carney, J. M.; Starke-Reed, P. E.; Oliver, C. N.; Stadtman, E. R.; Floyd, R. A.; Markesbery, W. R. Excess Brain Protein Oxidation and Enzyme Dysfunction in Normal Aging and in Alzheimer Disease. *Proc. Natl. Acad. Sci. U. S. A* 1991, 88, 10540-10543.
- (30) Glosli, H.; Tronstad, K. J.; Wergedal, H.; Muller, F.; Svardal, A.; Aukrust, P.; Berge, R. K.; Prydz, H. Human TNF-Alpha in Transgenic Mice Induces Differential Changes in Redox Status and Glutathione-Regulating Enzymes. *FASEB J.* 2002, 16, 1450-1452.
- (31) Thannickal, V. J.; Fanburg, B. L. Reactive Oxygen Species in Cell Signaling. *Am. J. Physiol Lung Cell Mol. Physiol* 2000, 279, L1005-L1028.
- (32) Adler, V.; Yin, Z.; Tew, K. D.; Ronai, Z. Role of Redox Potential and Reactive Oxygen Species in Stress Signaling. *Oncogene* 1999, 18, 6104-6111.
- (33) Sen, C. K. Redox Signaling and the Emerging Therapeutic Potential of Thiol Antioxidants. *Biochem. Pharmacol.* 1998, 55, 1747-1758.
- (34) Comhair, S. A.; Erzurum, S. C. Antioxidant Responses to Oxidant-Mediated Lung Diseases. *Am. J. Physiol Lung Cell Mol. Physiol* 2002, 283, L246-L255.
- (35) Laskin, D. L.; Pendino, K. J. Macrophages and Inflammatory Mediators in Tissue Injury. *Annu. Rev. Pharmacol. Toxicol.* 1995, 35, 655-677.
- (36) Muzykantov, V. R. Delivery of Antioxidant Enzyme Proteins to the Lung. *Antioxid. Redox. Signal.* 2001, 3, 39-62.
- (37) Davis, J. M. Superoxide Dismutase: a Role in the Prevention of Chronic Lung Disease. *Biol. Neonate* 1998, 74 Suppl 1, 29-34.
- (38) Noor, R.; Mittal, S.; Iqbal, J. Superoxide Dismutase--Applications and Relevance to Human Diseases. *Med. Sci. Monit.* 2002, 8, RA210-RA215.
- (39) Holley, J. A.; Janssen, Y. M.; Mossman, B. T.; Taatjes, D. J. Increased Manganese Superoxide Dismutase Protein in Type II Epithelial Cells of Rat Lungs After Inhalation of Crocidolite Asbestos or Cristobalite Silica. *Am. J. Pathol.* 1992, 141, 475-485.
- (40) Rahman, I.; MacNee, W. Oxidative Stress and Regulation of Glutathione in Lung Inflammation. *Eur. Respir. J.* 2000, 16, 534-554.
- (41) Li, X. Y.; Donaldson, K.; Rahman, I.; MacNee, W. An Investigation of the Role of Glutathione in Increased Epithelial Permeability Induced by Cigarette Smoke in Vivo and in Vitro. *Am. J. Respir. Crit Care Med.* 1994, 149, 1518-1525.
- (42) Puglia, C. D.; Powell, S. R. Inhibition of Cellular Antioxidants: a Possible Mechanism of Toxic Cell Injury. *Environ. Health Perspect.* 1984, 57, 307-311.

- (43) Li, X. Y.; Gilmour, P. S.; Donaldson, K.; MacNee, W. Free Radical Activity and Pro-Inflammatory Effects of Particulate Air Pollution (PM10) in Vivo and in Vitro. *Thorax* 1996, 51, 1216-1222.
- (44) Sagai, M.; Ichinose, T.; Oda, H.; Kubota, K. Studies on Biochemical Effects of Nitrogen Dioxide. II. Changes of the Protective Systems in Rat Lungs and of Lipid Peroxidation by Acute Exposure. *J. Toxicol. Environ. Health* 1982, 9, 153-164.
- (45) Rahman, I.; MacNee, W. Regulation of Redox Glutathione Levels and Gene Transcription in Lung Inflammation: Therapeutic Approaches. *Free Radic. Biol. Med.* 2000, 28, 1405-1420.
- (46) Gius, D.; Botero, A.; Shah, S.; Curry, H. A. Intracellular Oxidation/Reduction Status in the Regulation of Transcription Factors NF-KappaB and AP-1. *Toxicol. Lett.* 1999, 106, 93-106.
- (47) Rahman, I.; MacNee, W. Role of Transcription Factors in Inflammatory Lung Diseases. *Thorax* 1998, 53, 601-612.
- (48) Baeuerle, P. A.; Henkel, T. Function and Activation of NF-Kappa B in the Immune System. *Annu. Rev. Immunol.* 1994, 12, 141-179.
- (49) Rahman, I. Inflammation and the Regulation of Glutathione Level in Lung Epithelial Cells. *Antioxid. Redox. Signal.* 1999, 1, 425-447.
- (50) Christman, J. W.; Blackwell, T. S.; Juurlink, B. H. Redox Regulation of Nuclear Factor Kappa B: Therapeutic Potential for Attenuating Inflammatory Responses. *Brain Pathol.* 2000, 10, 153-162.
- (51) Christman, J. W.; Lancaster, L. H.; Blackwell, T. S. Nuclear Factor Kappa B: a Pivotal Role in the Systemic Inflammatory Response Syndrome and New Target for Therapy. *Intensive Care Med.* 1998, 24, 1131-1138.
- (52) Barnes, P. J.; Karin, M. Nuclear Factor-KappaB: a Pivotal Transcription Factor in Chronic Inflammatory Diseases. *N. Engl. J. Med.* 1997, 336, 1066-1071.
- (53) Julkunen, I.; Sareneva, T.; Pirhonen, J.; Ronni, T.; Melen, K.; Matikainen, S. Molecular Pathogenesis of Influenza A Virus Infection and Virus-Induced Regulation of Cytokine Gene Expression. *Cytokine Growth Factor Rev.* 2001, 12, 171-180.
- (54) Xiao, G. G.; Wang, M.; Li, N.; Loo, J. A.; Nel, A. E. Use of Proteomics to Demonstrate a Hierarchical Oxidative Stress Response to Diesel Exhaust Particle Chemicals in a Macrophage Cell Line. *J. Biol. Chem.* 2003, 278, 50781-50790.
- (55) Victorin, K. Review of the Genotoxicity of Ozone. *Mutat. Res.* 1992, 277, 221-238.
- (56) Vagaggini, B.; Taccola, M.; Conti, I.; Carnevali, S.; Cianchetti, S.; Bartoli, M. L.; Bacci, E.; Dente, F. L.; Di Franco, A.; Giannini, D.; Paggiaro, P. L. Budesonide Reduces Neutrophilic but Not Functional Airway Response to Ozone in Mild Asthmatics. *Am. J. Respir. Crit Care Med.* 2001, 164, 2172-2176.

- (57) Samet, J. M.; Hatch, G. E.; Horstman, D.; Steck-Scott, S.; Arab, L.; Bromberg, P. A.; Levine, M.; McDonnell, W. F.; Devlin, R. B. Effect of Antioxidant Supplementation on Ozone-Induced Lung Injury in Human Subjects. *Am. J. Respir. Crit Care Med.* 2001, 164, 819-825.
- (58) Fievez, L.; Kirschvink, N.; Dogne, S.; Jaspar, F.; Merville, M. P.; Bours, V.; Lekeux, P.; Bureau, F. Impaired Accumulation of Granulocytes in the Lung During Ozone Adaptation. *Free Radic. Biol. Med.* 2001, 31, 633-641.
- (59) Pryor, W. A.; Church, D. F. Aldehydes, Hydrogen Peroxide, and Organic Radicals As Mediators of Ozone Toxicity. *Free Radic. Biol. Med.* 1991, 11, 41-46.
- (60) Long, N. C.; Suh, J.; Morrow, J. D.; Schiestl, R. H.; Murthy, G. G.; Brain, J. D.; Frei, B. Ozone Causes Lipid Peroxidation but Little Antioxidant Depletion in Exercising and Nonexercising Hamsters. *J. Appl. Physiol* 2001, 91, 1694-1700.
- (61) McBride, D. E.; Koenig, J. Q.; Luchtel, D. L.; Williams, P. V.; Henderson, W. R., Jr. Inflammatory Effects of Ozone in the Upper Airways of Subjects With Asthma. *Am. J. Respir. Crit Care Med.* 1994, 149, 1192-1197.
- (62) Corradi, M.; Alinovi, R.; Goldoni, M.; Vettori, M.; Folesani, G.; Mozzoni, P.; Cavazzini, S.; Bergamaschi, E.; Rossi, L.; Mutti, A. Biomarkers of Oxidative Stress After Controlled Human Exposure to Ozone. *Toxicol. Lett.* 2002, 134, 219-225.
- (63) Rivas-Arancibia, S.; Vazquez-Sandoval, R.; Gonzalez-Kladiano, D.; Schneider-Rivas, S.; Lechuga-Guerrero, A. Effects of Ozone Exposure in Rats on Memory and Levels of Brain and Pulmonary Superoxide Dismutase. *Environ. Res.* 1998, 76, 33-39.
- (64) Plopper, C. G.; Duan, X.; Buckpitt, A. R.; Pinkerton, K. E. Dose-Dependent Tolerance to Ozone. IV. Site-Specific Elevation in Antioxidant Enzymes in the Lungs of Rats Exposed for 90 Days or 20 Months. *Toxicol. Appl. Pharmacol.* 1994, 127, 124-131.
- (65) Fakhrzadeh, L.; Laskin, J. D.; Gardner, C. R.; Laskin, D. L. Superoxide Dismutase-Overexpressing Mice Are Resistant to Ozone-Induced Tissue Injury and Increases in Nitric Oxide and Tumor Necrosis Factor-Alpha. *Am. J. Respir. Cell Mol. Biol.* 2004, 30, 280-287.
- (66) Zeidler, P. C.; Castranova, V. Role of Nitric Oxide in Pathological Responses of the Lung to Exposure to Environmental/Occupational Agents. *Redox. Rep.* 2004, 9, 7-18.
- (67) Ghio, A. J.; Richards, J. H.; Carter, J. D.; Madden, M. C. Accumulation of Iron in the Rat Lung After Tracheal Instillation of Diesel Particles. *Toxicol. Pathol.* 2000, 28, 619-627.
- (68) Gurgueira, S. A.; Lawrence, J.; Coull, B.; Murthy, G. G.; Gonzalez-Flecha, B. Rapid Increases in the Steady-State Concentration of Reactive Oxygen Species in the Lungs and Heart After Particulate Air Pollution Inhalation. *Environ. Health Perspect.* 2002, 110, 749-755.

- 
- (69) Han, J. Y.; Takeshita, K.; Utsumi, H. Noninvasive Detection of Hydroxyl Radical Generation in Lung by Diesel Exhaust Particles. *Free Radic. Biol. Med.* 2001, 30, 516-525.
- (70) Stringer, B.; Imrich, A.; Kobzik, L. Flow Cytometric Assay of Lung Macrophage Uptake of Environmental Particulates. *Cytometry* 1995, 20, 23-32.
- (71) Goldsmith, C. A.; Imrich, A.; Danaee, H.; Ning, Y. Y.; Kobzik, L. Analysis of Air Pollution Particulate-Mediated Oxidant Stress in Alveolar Macrophages. *J. Toxicol. Environ. Health A* 1998, 54, 529-545.
- (72) Brunekreef, B.; Holgate, S. T. Air Pollution and Health. *Lancet* 2002, 360, 1233-1242.
- (73) Dockery, D. W.; Pope, C. A., III; Xu, X.; Spengler, J. D.; Ware, J. H.; Fay, M. E.; Ferris, B. G., Jr.; Speizer, F. E. An Association Between Air Pollution and Mortality in Six U.S. Cities. *N. Engl. J. Med.* 1993, 329, 1753-1759.
- (74) Peters, A.; Dockery, D. W.; Muller, J. E.; Mittleman, M. A. Increased Particulate Air Pollution and the Triggering of Myocardial Infarction. *Circulation* 2001, 103, 2810-2815.
- (75) Jimenez, L. A.; Thompson, J.; Brown, D. A.; Rahman, I.; Antonicelli, F.; Duffin, R.; Drost, E. M.; Hay, R. T.; Donaldson, K.; MacNee, W. Activation of NF-KappaB by PM(10) Occurs Via an Iron-Mediated Mechanism in the Absence of IkappaB Degradation. *Toxicol. Appl. Pharmacol.* 2000, 166, 101-110.
- (76) Bowler, R. P.; Crapo, J. D. Oxidative Stress in Allergic Respiratory Diseases. *J. Allergy Clin. Immunol.* 2002, 110, 349-356.
- (77) Kooter, I. M.; Pennings, J. L. A.; Gerlofs-Nijland, M. E.; Cassee, F. R. Gene Expression Pattern in Spontaneously Hypertensive Rats Exposed to Urban Particulate Matter (EHC-93). *Inhalation Toxicology* 2004.