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# Pulmonary inflammation and systemic effects of model and ambient particles

## Dissertation

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## Mathematisch-Naturwissenschaftliche Fakultät der Heinrich-Heine-Universität Düsseldorf

**To my family and in memory of my dear mother Sarie**

**Gez.**

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***The teacher who is indeed wise does not bid you to enter the house of his wisdom but rather leads you to the threshold of your mind.***

**Kahlil Gibran**

**Contents**

## Abstract

**Introduction:** Ambient particulate matter (PM) exposure has been implicated in various epidemiological studies to be responsible for adverse health effects ( Daniels et al, 2000; Pope et al, 2002). These studies estimated that an increase of 10 μg/$m^3$ $PM^{2.5}$ resulted in mortality increase of 1.4 % while respiratory diseases such as bronchitis or asthma exacerbation increase by much as 4 % (WHO, 1999). Various hypotheses exist, but no clear biological mechanism or pathway is at hand to explain what cause these health effects observed in epidemiological studies. Toxicological and epidemiological evidence suggest that fine ($PM_{2,5}$) and ultrafine ($PM_{0,1}$) fraction is responsible for these adverse effects, however there seems to be no agreement. The purpose of this study was to test if the previous autonomic effects can occur in a system that preserves the structural integrity of the lung and allows to study particle uptake as well as particle induced release of inflammatory and vaso-active substances.
**Methods:** The isolated perfused rat lung (IPRL) is suitable for this goal, since this system allows us to i) exclude systemic anti-oxidant back-up from the perfusing blood, allows to study inflammation using PMN migration into tissue and alveolar space, iii) retains structural integrity necessary to study the effect of ultrafine patticles and iv) to sample perfusate that can be investigated for direct biological activity and the presence of particles. Experiments were designed to elucidate inflammation as modulator of lung permeability, translocation of particles and as a generator of vaso-active substances (all induced by instillation by particles). Particle translocation was monitored by radioactivity of the particles (Iridium) and not by any attached radioactive label. Other models used for these studies were the isolated perfused rat heart and H9C2 cardiomyocyte.
**Results:** No translocation of Iridium (Ir) particles (17-20 nm) was detected in normal negative pressure perfused lungs. However lungs pre-treated with histamine on the endothelial side (1,uM) or $H_20_2$ (0.5 mM) in the alveolar lumen showed small amounts of radioactivity in the single pass perfusate after a significant lag-time. This particle translocation coincided with increased permeability for DTPA in the histamine perfused and $H_20_2$ treated lungs. No translocation of Ir particles (17-20 nm) was detected in normal positive pressure perfused lungs. Even lungs pre-treated with histamine on the endothelial side (1μM) or $H_20_2$ (0.5 mM) in the alveolar lumen, showed no amounts of radioactivity in the recirculating perfusate after a significant lag-time. Addition of PMN in perfusate with PMN activators did not reveal any Ir particles in the perfusate. Additionally, we establish an ex-vivo inflammatory lung model that can be used in variety of pulmonary investigations. Along with it, we could successfully label human PMN's to be used in an isolated perfused rat lung model. Viewing the effect that ambient particles have on SHR rats, our data showed that pre-treatment of SHR rats with ambient particles and LPS does affect the ability of the isolated heart to recover after an ischaemic insult caused by coronary occlusion, although no difierence was observed.
**Conclusion:** We conclude that effects may be caused by soluble components in the PM preparation. This is supported by our cellular studies in cardiomyocytes where we observed an effect of PM end metals on intracellular calcium channels. Although these data do not allow definitive conclusions on the exact mechanism and the importance of systemic translocation of UFP as a mechanism in adverse effects of PM, we do confirrn that ultrafine particles can translocate from the lung into the circulation using the isolated perfused rat lung upon pharmacological mediation. Permeability of the lung barrier to ultrafine particles seemsto be controlled both at the epithelial and endothelial level and conditions that affect this barrier function such as inflammation may affect translocation of UFP.

# Zusammenfassung

**Einleitung:** In verschiedenen epidemiologischen Studien wurde Kontakt mit Schwebstoffen (nachfolgend „PM“ – von „particulate matter“) in der Umgebungsluft mit gesundheitsschädigenden Wirkungen in Verbindung gebracht (Daniels et al, 2000; Pope et al, 2002). Diese Studien haben geschätzt, dass eine 10 $\mu g/m^3$ $PM^{2.5}$ Erhöhung der Schwebstoffmenge zu einer 1.4%-igen Mortalitätserhöhung führt und, dass bei Atemwegerkrankungen wie Bronchitis oder Asthma diese Mortalitätserhöhung auf bis zu 4% steigen kann (WHO, 1999). Es gibt verschiedene Hypothesen aber kein eindeutiger biologischer Mechanismus oder Signalweg, um diese in den epidemiologischen Studien beobachteten Auswirkungen zu erklären. Toxikologische und epidemiologische Befunde deuten darauf hin, dass feine ($PM_{2,5}$) bzw. Feinstpartikeln ($PM_{0,1}$) für diese gesundheitsschädigende Wirkungen verantwortlich sind; es besteht jedoch anscheinend keine Übereinstimmung. Der Zweck dieser Studie war zu prüfen, ob die vorherige autonomische Auswirkungen in einem System, das die strukturelle Intaktheit der Lunge beibehält, auch auftreten können. Dieses System muss auch die Untersuchung von Teilchenaufnahme und teilchenbedingte Abgabe von Entzündungs- und vasoaktiven Substanzen ermöglichen.
**Methoden:** Die isolierte, durchschwemmte Rattenlunge (nachfolgend „IPRL“ – von „isolated perfused rat lung“) ist hierfür geeignet, da dieses System folgendes ermöglicht: i) der Ausschluss der systemischen Antioxidationsmittel-Aktivierung in dem durchschwemmenden Blut, ii) die Untersuchung der Entzündung anhand der Leukozytmigration ins Gewebe und in den alveolaren Bereich, iii) die erforderliche Beibehaltung der strukturellen Intaktheit, um die Auswirkung von Feinstpartikeln untersuchen zu können und iv) die Entnahme von Perfusatproben, die auf direkte biologische Aktivität und die Anwesenheit von Partikeln untersucht werden können. Experimente wurden konzipiert, um festzustellen, ob Entzündung als einen möglichen Veranlasser der Lungendurchlässigkeit und Partikeltranslokation und als einen Erzeuger von vasoaktiven Substanzen fungiert (alle über Partikeleinflößung eingeleitet). Partikeltranslokation wurde über Partikelradioaktivität (Iridium) und nicht über eine angebrachte radioaktive Markierung überwacht. Andere für diese Studien benutzten Modellen waren ein isoliertes, durchschwemmtes Rattenherz und H9C2 Kardiomyozyt.
**Ergebnisse:** Translokation von Iridiumpartikeln (Ir-Partikeln) (17 – 20 nm) wurde in normalen, bei Unterdruck durchschwemmten Lungen nicht festgestellt. Jedoch wurden bei Lungen, die auf der Endothelseite mit Histamin (1, µM) oder in den alveolaren Lumen mit $H_20_2$ (0.5 mM) vorbehandelt wurden, nach einer signifikanten Verzögerung kleine Mengen an Radioaktivität in dem einmalig verwendeten Perfusat festgestellt. Diese Partikeltranslokation wurde zusammen mit erhöhter Durchlässigkeit für DTPA in den mit Histamin durchschwemmten und mit $H_20_2$ behandelten Lungen festgestellt. Translokation von Ir-Partikeln (17 – 20 nm) wurde in normalen, bei Überdruck durchschwemmten Lungen nicht festgestellt. Sogar bei Lungen, die auf der Endothelseite mit Histamin (1µM) oder in den alveolaren Lumen mit $H_20_2$ (0.5 mM) vorbehandelt wurden, wurde nach einer signifikanten Verzögerung keine Radioaktivität in dem umlaufenden Perfusat festgestellt. Der Zusatz von Leukozyten zum Perfusat mit Leukozytenaktivierungsmitteln deckte keine Ir-Partikeln im Perfusat auf. Darüber hinaus haben wir ein ex-vivo entzündetes Lungenmodell eingerichtet, das in einer Reihe von Lungenuntersuchungen verwendet werden kann. Hiermit konnten wir menschliche Leukozyten zur Nutzung im IPRL-Präparat erfolgreich markieren. Bei der Beobachtung der Auswirkung der Umgebungspartikeln auf SHR-Ratten, zeigten unsere Daten, dass die Vorbehandlung von SHR-Ratten mit Umgebungspartikeln und LPS eine Auswirkung auf der Erholungsfähigkeit eines isolierten Herzens nach einem durch Koronarverschluss verursachten ischämischen Insult hat, obwohl kein Unterschied beobachtet wurde.
**Schlussfolgerungen:** Wir sind zu der Schlussfolgerung gekommen, dass die Auswirkungen möglicherweise durch lösliche Komponenten in dem PM-Präparat verursacht werden

können. Dies wird durch unsere Zelluntersuchungen bei Kardiomyozyten, in dem wir eine Auswirkung auf intrazellulare Kalziumkanäle bei PM und Metallen beobachtet haben, unterstützt. Obwohl diese Daten keine eindeutige Schlussfolgerungen bezüglich des genauen Mechanismus und der Bedeutung von der systemischen Translokation von Feinstpartikeln als Mechanismus in negativen PM-Auswirkungen ermöglichen, bestätigen wir anhand der IPRL, dass unter pharmakologischem Eingriff, Feinstpartikeln von der Lunge in den Blutkreislauf translokieren können. Die Durchlässigkeit der Lungenmembran gegenüber Feinstpartikeln scheint sowohl auf Epithel- als auch auf Endothelebene geregelt zu sein und Bedingungen wie Entzündung, die eine Auswirkung auf diese Membranfunktion haben, möglicherweise auch eine Auswirkung auf die Translokation von Feinstpartikeln haben können.

# Abbreviations

| | |
|---|---|
| $^{13}C$ | element 13 carbon |
| $^{192}Ir$ | Iridium particles |
| ACE | Angiotensin Converting Enzyme |
| ALP | Alkaline Phosphatase |
| ATP | Adenosine triphosphate |
| CFSE | carboxyfluorescein diacetate, succinimidyl ester |
| COPD | Chronic Obstructive pulmonary disease |
| COX-2 | cyclooxygenase 2 |
| DTPA | diethylene triamine pentaacetic acid |
| e.g. | for example |
| EHC-93 | Ottowa dust (ambient particles) |
| EPR | Electron paramagnetic resonance also known as electron spin resonance |
| FAPGG | N-[3-(2-furyl) acryloyl]-L-phenylalanylglycylglycine |
| FMLP | N-formyl-methionyl-leucyl-phenylanaline |
| GM-CSF | Granulocyte Macrophage-Colony Stimulating Factor |
| $H_2O_2$ | Hydrogen Peroxide |
| HBSS | Hanks' balanced salt solution |
| i.e. | 'in other words' or 'in this case' |
| IL-8 | Interleukin-8 |
| IPL | Isolated Perfused Lung |
| IPRL | Isolated perfused Rat Lung |
| IU | International Units |
| LPS | Lipopolysaccharide |
| LVDP | Left Ventricular Developing Pressure |
| $MnO_2$ | Manganese dioxide |

| | |
|---|---|
| MPO | Myeloperoxidase |
| Nm | nanometer |
| Nox | Nitric oxide radical |
| OH. | Hydroxyl radical |
| PAH | Polycyclic aromatic hydrocarbon(s) |
| PBS | Phosphate buffered saline |
| PM | Particulate Matter |
| $PM_{10}$ $PM_{2,5}$ | Particulate Matter (diameter ) |
| PMN | Polymorphonuclear neutrophils |
| ROS | reactive Oxygen Species |
| SHR | Spontaneous Hypertensive Rats |
| $SO_2$ | sulfate dioxide |
| Tc-DTPA | $Tc^{99m}$-labeled diethylene triamine pentaacetic acid |
| TLR | Toll-like receptor |
| TNF$\alpha$ | Tumour necrosis factor$\alpha$ |
| UFP | ultra fine particles |

# CHAPTER 1

# INTRODUCTION

### 1.1 Adverse Health effects of ambient particulate matter (PM)

The average human inhales approximately around $3x10^8$ L of air in his life span. However the air we breathe is not clean and thus we breathe a lot of air pollutants, including gases such as $SO_2$, ozone, $NO_x$ and ambient particles. Whereas in the seventies and eighties the oxidant gases received considerable attention, since 1993 (Dockery et al, 1993) the exposure to airborne particulate matter (PM) has gained enormous interest. PM is a complex mixture of particles ranging in size over five orders of magnitude that range from molecular dimensions to the size that are distinguishable with the naked eye. Ambient particles contain a range of particle types that result both from man-made and natural sources. The biologically relevant size fraction ranges from 10 µm (PM10), through its fine fraction smaller than 2.5 µm ($PM_{2.5}$) down to the ultrafine particle range (< 100 nm). Ultrafine particles are generated mainly from combustion processes, gas to particle conversion. For this fraction also the term nanoparticles is frequently encountered, but it needs emphasis that this group also contains synthetical and engineered particles that are very different form combustion nanoparticles (Borm et al, 2004). Various physical characteristics of fine, coarse and ultrafine particles have been investigated in experimental models and have demonstrated that both particle size and surface area are linked to their biological activity (for review: Borm & Kreyling, 2004; Oberdorster, 2001; Donaldson et al, 2002). In addition experimental studies have shown the importance of a number of constituents, including transition metals (Schaumann et al, 2004; Ghio et al, 2002) and endotoxins (Schins et al, 2003) in the adverse effects of ambient particles.

A large set of epidemiological studies has now established the relationship between exposure to ambient particulate matter (PM) and various acute adverse effects patients with pulmonary or cardiovascular diseases (Pope et al, 2004; Dockery et al, 1993 ; Peters et al., 2004) and is also related to premature mortality ( Dominici et al, 2000). Typically, relative risks of exposed and vulnerable groups are expressed as the increased mortality or morbidity at an annual increase of $10\mu g/m^3$ (see also table 1). In the next paragraphs we will detail more specifically on the risks encountered by patients with pulmonary diseases (1.1.1) or cardiovascular disorders (1.1.2).

### 1.1.1 Pulmonary effects of ambient particulate matter (PM)

Numerous epidemiological studies have shown that air pollution increases morbidity and mortality among those with existing respiratory disease (review: Pope et al, 2004). Local air pollution resulted in health effects among residents with asthma, as well as other respiratory diseases or cardiovascular disease. In addition, PM is now suggested to be related to lung cancer (Beeson et al, 1998; Pope et al, 2002). Children seem to be vulnerable group and the respiratory effects on children are observed with emergency visits, missed school days and bronchitis as out comes (review: Brunekreef). Epidemiological studies have consistently shown an association of the above adverse effects on sensitive parts of the population with slight increases in concentration of ambient particular matter. Within a short-lag time of one or two days following an increase in PM there are:- 1) increases in all-cause mortality; 2) significant increases in attacks of asthma and increased usage of asthma medication ;3) increased deaths in COPD patients, 4) exacerbations of COPD, 5) increased deaths and hospitalizations for cardiovascular disease. The adverse cardiovascular effects associated with increases in PM are well-documented. Panel studies have documented associations between elevated levels of particles and 1) onset of myocardial infarction, 2) increased heart rate and 3) decreased heart rate variability (Utell et al, 2002). Chamber studies on human volunteers exposed to concentrated airborne particles (CAPs) have also shown increased lung inflammation and altered brachial artery diameter in relation to increased exposure (Brook et al, 2002; Holgate et al, 2003)

Table 1.1 Estimated effects of an increase in annual PM2.5 concentration (10 μg/$m^3$) on the adjusted Mortality Relative Risk (RR) of specific disease groups. (Source: Pope, 2002).

| | | | |
|---|---|---|---|
| **Caused of Mortality** | **1979-1983** | **1999-2000** | **Average** |
| All cause | 1.04 (1.01-1.08) | 1.06 (1.02-1.10) | 1.06 (1.02-1.11) |
| Cardiopulmonary | 1.06 (1.02-1.10) | 1.08 (1.02-1.14) | 1.09 (1.03-1.16) |
| Lung Cancer | 1.08 (1.01-1.16) | 1.13 (1.04-1.22) | 1.14 (1.04-1.23) |
| All other cause | 1.01 (0.97-1.05) | 1.01 (0.97-1.06) | 1.01 (0.95-1.06) |

### 1.1.2 Cardiovascular effects

Both in Europe and the United States disease of the heart and blood vessels is the major cause of death (Guidicelli and Chanudet 2004). Diseases of blood vessels are a major cause of premature disability and death. Heart attacks and strokes are the most obvious consequence of damaged arteries and increased clotting of blood. A number of possible

cardiovascular disease outcomes and endpoints have been considered in epidemiological research. These include all-cause mortality and endpoints specific for cardiovascular mortality and morbidity, including sudden out-of-hospital death, acute myocardial infarction, stroke, congestive heart failure, peripheral vascular disease, congenital heart disease and cardiomyopathy. In the USA around 11.2 million people suffer from ischemic heart disease and 1.5 million suffer acute myocardial infarction each year. Along that it is predicted that one-third of the population have some form of hypertension and the cause of 90-95 percent of these cases is unknown. The risk is further demonstrated in relation to both short- and long-term exposure to PM.

The increased risk for the above cardiovascular events due to increased concentrations of ambient particulate matter are now more and more demonstrated in epidemiological and panel studies (e.g. Peters et al, 2004, Delfino et al, Review 2005). Quite recently the issue has got beyond particle and pulmonary toxicology and has attracted considerable attention among specialists in cardiovascular diseases (Pekkanen et al 2002, Brauer et al 2001, Brook et al 2004, Brunekreef and Holgate, 2002). Cardiovascular effects noted from recent studies include disruption of autonomic nervous system activity (Pope 1999), arterial vasoconstriction (Brook 2002, Nurkiewicz et al, 2004) and relaxation (Bagate et al, 2004) , cardiac arrhythmia's in patients with defibrillators (Peters 2000), cardiac events such as myocardial infarction (Peters 2001, Peters et al, 2004) that required hospitalisation and exacerbation of the ST-segment changes in experimental models and humans after myocardial infarction (Godleski 2000, Pekkanen et al, 2002 ).

**Table 1.2.**

Overview of human studies indicating cardiovascular effects of ambient PM

| Effect | Study conditions | Authors |
|---|---|---|
| Vasoconstriction | In vivo measure of brachial artery diameter (PM2.5 + $O_3$), 25 volunteers | Urch et al, 2004<br>Brook et al, 2002 |
| Blood pressure | Ambient particulate matter effect on heart rate and blood pressure with patient in 3 cities | Ibald-Mulli et al, 2004 |
| Heart rate variability | Concentrated ambient coarse particle (whole body exposure), Asthmatic and | Gong et al, 2004,<br>Chan et al, 2004,<br>Ghio and Huang 2004 |

| | | |
|---|---|---|
| | Healthy Volunteers | Devlin et al, 2003 |
| Arrythmia's | Out door air pollutants. Patients with implantable Cardioverter Defibrillators | Vedal et al, 2004<br>Rich et al, 2004 Devlin, Utell, Frampton (2002) |
| Vasoconstriction | Diesel exposure affects bloodflow and regulation in forearm model | Mills et al, (2005) |
| Myocardial infarction | Exposure to traffic and the onset of myocardial infarction<br>Extension of ST segment upon exercise | Peters, 2004 , 2001<br>Pekkanen et al, 2002 |
| Atherosclerosis | Long term exposure to ambient PM up to 2.5 µm | Kunzli, 2005 (EHP) |

A common element in most chronic heart diseases is poor blood flow to the heart muscle, usually because of the atherosclerotic plaques in the coronary vessels and the accumulation of platelets, leukocytes and other deposits that block the flow of blood to the heart muscle. It is postulated that substances in PM , such as polycyclic aromatic hydrocarbons (PAHs) may contribute to the heart disease by damaging the endothelial barrier in the vascular system, activating leukocytes and platelets and initiating the formation of atherosclerotic plaques and stimulating an inflammatory response (Curfs et al, 2004). However, the kinetics and absorption from PAHs after inhalation of PM or dietary intake of PAHs may be completely different, and therefore the hazard extrapolation needs more study. Another well established risk factor for cardiovascular disease is blood pressure. Although PM has been shown to affect acute vascular contraction (Batalha et al, 2002; Brook et al, 2002: Nurkiewicz et al, 2004) and relaxation (Bagate et al, 2004) in various experimental models, it is still unclear whether this also affects chronic whole body blood pressure (Chang et al, 2004; Wichers et al, 2004) and how this impacts on cardiovascular events at later stage.

Systemic inflammation is also regarded as a driver or catalyst to many adverse cardiovascular effects. There is human evidence of systemic inflammation following exposure PM, as ilustrated by elevated C-reactive protein, blood leukocytes, platelets, fibrinogen and increased plasma viscosity (Seaton 1995, Seaton 1999, Peters 1997; Ghio & Devlin, 2001). Systemic inflammation is also relevant with regard to atherosclerosis, which is the underlying cause of acute coronary syndrome, being the main cause of cardiovascular morbidity and mortality. Atherogenesis is an inflammatory process, initiated via endothelial

injury and producing systemic markers of inflammation that are risk factors for myocardial and cerebral infarction (Libby et al, 2002). Repeated exposure to PM may, by increasing systemic inflammation, exacerbate the vascular inflammation of atherosclerosis and promote plaque development or rupture. Thus inflammation in the lungs is suggested to explain both the local pulmonary and systemic cardiovascular effects of PM (Donaldson et al, 2004). This concept is illustrated in Fig.1

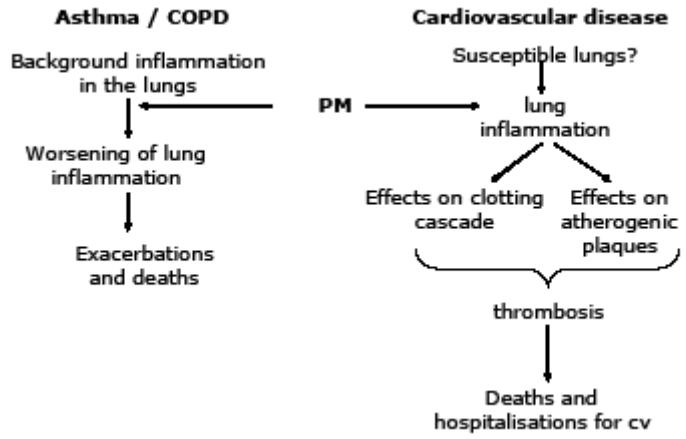



Fig. 1.1 An illustration that shows inflammation as a key-event that connects pulmonary and cardiovascular effects of PM inhalation. Adapted from Donaldson et al, (2004)

Apart from particulate matter (PM) also carbon monoxide, ozone, NOx and $SO_2$ has been associated with increased hospitalisation and mortality due to cardiovascular disease (Pope 2004, Pope 2002, Samet, 2000). Air pollution of total suspended particulates and $SO_2$ were associated with an increase in blood pressure and this could be related to a change in cardiovascular autonomic control (Ibaldi-Mulli et al, 2001). Carbon monoxide has been shown to cause exacerbations of cardiovascular disease in humans (Brook et al 2004). Also the original observations on brachial diameter reduction in humans upon exposure to CAPS (153 $\mu g/m^3$) were done in the presence of ozone (120 ppb). Therefore an interaction with or direct effect of ozone cannot be excluded (Brook et al, 2002; Urch et al, 2004).

### 1.2 Mechanisms of cardiovascular effects of PM

As we indicated in the previous paragraph inflammation is currently suggested as one of the mechanisms linking pulmonary and systemic effects of inhaled particles. This concept is attractive for reasons of biological plausibility providing amplification and signalling route to the heart and vascular system. However, recent studies have indicated that alternative, parallel pathways may play a role. These mechanisms are briefly reviewed in the next

paragraphs and discriminated into direct and indirect pathways. Inflammation, as indicated in Fig. 1 falls under the indirect pathways.

### 1.2.1 Indirect pathways

Possible indirect effects of PM may occur via pulmonary oxidative stress and inflammation (see also Fig. 1). This subsequently might lead to a systemic inflammatory state which can activate blood coagulation pathways (Nemmar et al, 2003; Ghio & Devlin, 2001), impair most of vascular function (Bagate et al, 2004 and 2005; Nurkiewicz et al, 2004 and 2006) and accelerate atherosclerosis (Suwa et al, 2002; Chen et al, 2005).

Toxicological studies have shown that oxidative stress is a central mechanism that may be responsible for the inflammatory effects of PM causing pulmonary responses (Oberdorster 2001; Donaldson et al, 2003). On the other hand, inflammation is known to amplify the burden of reactive oxygen species to the lung, and thereby causes a persistent oxidative stress. PM contains several components that are able to generate oxidative stress when the particles deposit on the lung surface:

1) Transition metals Formation of hydroxyl radicals (OH●), generated by ionic iron and other transiton metals via the Fenton-reaction has been implicated in the oxidative stress and inflammatory effects of PM. Other transition metals such as iron, vanadium and copper are also found in occur in PM and generate OH● (Ghio et al, 1999; Prahalad, 2001, Shi et al 2003a). Electron paramagnetic resonance (EPR) spectroscopy using a spintrap has been used to directly demonstrate the generation of OH● by PM in the presence of $H_2O_2$ (Shi et al, 2003a and b). In these studies OH● formation is prevented by the addition of transition metal chelator desferoxamine (Shi et al, 2003; Knaapen et al, 2002). In addition, OH●-formation is a better descriptor than mass in the inflammatory response of humans after bronchial instillation of different PM2.5 samples (Schaumann et al, 2004)
2) Nanoparticle surfaces. Surface area of particles is known to be a major factor in driving inflammation (Tran et al, 2000; Oberdorster, 2001). Nanoparticles of low toxicity, low solubility materials have a very high surface area per unit mass. They are also highly inflammogenic by virtue of this high surface area and oxidative stress, generated through mechanisms that are not well understood, appears to play a role.

PM can contain very high numbers of combustion derived nanoparticles as singlet particles but also as aggregates; these aggregates can be aerodynamically larger than the classical definition of a nanoparticle, i.e. they can be larger than 100 nm. However, nanoparticles in aggregates still express toxicity consistent with their geometric surface area and not in relation to their surface area as deduced from their aerodynamic diameter as an aggregate.

3) Organics. PM contain organic molecules that have the ability to generate free radicals and cause oxidative stress (Dellinger et al, 2001). These organics include PAHs, n-alkanes nitro-PAHs and quinones formed by combustion or derived from unburnt fuel. Interaction of these organics with enzyme systems can results in oxidative stress via the production of oxidants such as superoxide anion. These also have the ability to activate oxidative stress-responsive signalling pathways in cells, leading to release of pro-inflammatory chemokines ( IL-8) or growth factors such as GM-CSF (Boland et al, 2000).

4) Endotoxin. Endotoxins are biological components, present in some particle samples that are released from the outer membrane of gram-negative bacteria. Chemically they are defined as lipopolysaccharides (LPS) and found in variable amounts in PM from various locations (Heinrich et al, 2003). Studies indicate that the PM endotoxin can be a major component in stimulating cells, especially macrophages to produce inflammatory mediators. Endotoxins are commonly more present in the coarse fraction ($PM_{10}$ and $PM_{2.5-10}$) than in the fine fraction (Monn & Becker, 1999; Schins et al, 2004). Endotoxins are known to induce a series of inflammatory response that are controlled by a set of receptors (TLR, TNF-receptors) that show a marked inter-individual hetergenecity (Kleeberger et al, 2001).

Seaton et al, (1995) hypothesized that particulate air pollution may provoke alveolar inflammation, resulting in the release of potentially harmful cytokines, increased blood coagulation and systemic inflammation, which may aggravate existing cardiovascular events. Later studies in humans exposed to diesel exhaust particulates (Salvi et al, 1999) or $PM_{2.5}$ by bronchial instillation (Ghio et al, 2002; Schaumann et al, 2004) showed a marked pulmonary inflammatory response in healthy humans. Both in human and animal studies increased migration of immature PMN from the bone marrow into the blood was noted as well as increased blood cytokines upon exposure to PM (Suwa et al, 2002; van Eeden et al, 2001).

An alternative mechanism for indirect effects of PM is provided by the uptake of ultrafine particles through the olfactory epithelium, which is situated in the upper nasal region. This thin epithelial layer provides a shortcut for particles to enter the brain from the nasal space. This pathway was shown by Öberdorster et al (2004) using the $^{13}C$-isotope in ultrafine spark generated carbon particles, and more recently also with ultrafine gold and $MnO_2$ particles (Oberdorster et al, 2004; ATS abstract). The deposition of inhaled ultrafine particles in the respiratory tract is governed by diffusional processes. Recent pathological studies by Calderon-Garcidueneas et al in animals (2002) and human subjects (2004) living in highly polluted areas suggest that an extensive amount of these ultrafine particles translocate to the brain. In addition, these and other studies (Campbell et al, 2004) suggest that translocated nanoparticles may lead to an increased expression of inflammatory mediators such as COX-2 (Calderon-, 2004), brain levels of IL-1 and TNFα (Campbell et al, 2004) or a decrease of dopaminergic neurons (Block et al, 2004). It remains to be elucidated how these events in the CNS can affect peripheral and systemic function.

### 1.2.2 Direct pathways of cardiovascular effects of PM

The biological mechanisms linking air pollution to heart disease may also involve direct effects of pollutants on the cardiovascular system, lung receptors, blood and/or indirect effects such pulmonary oxidative stress and inflammatory responses. Theoretically, direct effects can occur via particles or agents that cross the pulmonary epithelium into the circulation and reach the heart or vasculature. This may occur for the ultrafine particle fraction of PM and/or the soluble constituents of PM. In addition, PM or components may activate pulmonary receptors that have a direct connection with autonomic innervation of the heart (review: Widdicombe, 2003). This phenomenon has been described for other pollutants such as $SO_2$ (Tunnicliffe et al, 2001). The autonomic nervous system may also activate changes in blood viscosity, heart rate, and heart rate variability and thus may increase the likelihood of cardiac death (Nolan 1998). However, little *in vivo* evidence has been found that PM uses such pathways. On the other hand *in vitro* studies have demonstrated that PM or PM constituents such as metals and PAHs can affect autonomic regulation in preparations such as aortic rings, mesenteric veins (Bagate et al, 2004) and isolated cardiac muscle (Sakakibara et al 1994). These direct effects of air pollution represent a theoretical explanation for the occurrence of the rapid cardiovascular responses, such as increased in myocardial infarction with several hours of participation in traffic (Peters et al, 2004). The probability that this occurs is however dependent on the translocation of particles and low constituents into the circulation.

As indicated above, the ultrafine particle fraction of PM may be able to traverse the epithelial barrier and gain access to the pulmonary interstitium. Particles in the interstitium are unlikely to be cleared and so the dose builds up (Ferin et al, 1992; Nikula, 1997; Borm & Kreyling, 2004). In the interstitium the NP can activate cells leading to interstitial inflammation. In the interstitium the particles are very close to the blood and experimentally NP has been shown to gain access to the blood. It has been shown that diesel particles, Technetium –labelled carbon particles (Nemmar 2002), ultrafine Iridium particles (Kreyling et al, 2002), albumin nanocolloid particles ( Nemmar et al, 2001 ) and radiolabelled latex nanoparticles (Brooking et al, 2001) can enter the blood stream after inhalation. If this is confirmed for environmental NP, then we may postulate that blood borne nanoparticles may interact with endothelial cells and clotting proteins so enhancing thrombogenesis. Additionally they could interact directly with the endothelium overlying atherogenic plaques and could even gain access to plaques. This could cause activation of cells in the plaques and accelerate plaque growth or rupture, making a direct contribution to acute coronary syndrome. The mechanism by which Nanoparticles find their way across the cell membranes (trans-cellular transport) remains unknown but due to their particle size it is possible that they will not use the normal route of

endocytosis. On the other hand aggregates of primary particles, may easily reach diameters accessible for endocytosis (> 200 nm). Apart from endocytosis uptake via caveolae or clathrin-coated pits, pinocytosis or via adhesive interference may be possible pathways of uptake (Gumbleton, 2001).

Although particle translocation is a direct mechanism for the systemic effects of the (ultrafine) particle fraction, one needs to consider that the para-cellular translocation is dependent on the permeability of the different lung barriers. In turn these may be highly dependent on particle properties as well processes such oxidative stress and inflammation that may enhance or inhibit permeability. This concept is illustrated in the diagram in Fig. 2.

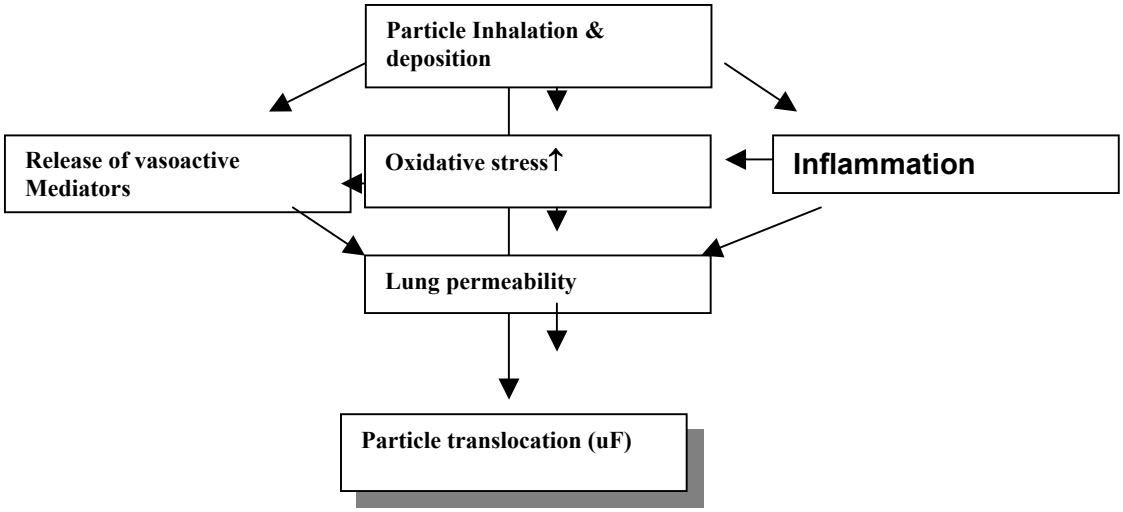



Fig. 2

Schematical illustration of particle-induced processes in the lung such as oxidative stress, inflammation and release of vasoactive substances (e.g. histamine) can affect epithelial and endothelial permeability. This permeability is thought to be a crucial process in the paracellular translocation of ultrafine particles.

### 1.2.3 Lung permeability

Lung permeability can be defined from different perspectives as well as methodological tools. Conventional measures for lung permeability include translocation of small and large weight substances with radioactive labels (DTPA, $^{13}C$), conventional dyes, trans-migration of neutrophils (intravital fluorescence microscopy, Kuhnle et al, 199), and migration of nanoparticles from the blood stream (Heckel et al, 2004). More specific measures of permeability of the lung capillary bed are available from temporal occlusion in lung perfusion models (Hamoir et al, 2003; Methods in Pulmonary Research, 1998). Since the mechanism of particle translocation so far remains to be elucidated, no objective choice from the available methods can be made. For any particle to cross the pulmonary barrier includes passing respiratory mucus, mucociliary clearance, the alveolar epithelium, the basement

membrane, pulmonary (defense) enzymes, interstitial leukocytes and macrophages (Niven ,1995) and finally the endothelium. For our understanding and the purpose of these studies we suggest that the rate of particle translocation in the lung is determined by two main barriers, i.e. the lung epithelium and the vascular endothelial layer separated by the interstitium. These barriers can be passed by two different mechanisms, including trans-cellular and para-cellular transport. This model is illustrated in Fig. 3. The alveolar epithelium and capillary endothelium both have high permeability to water, gases and lipophilic substances, the permeation of many hydrophilic substances of large molecular size and of ionic species is limited (Sayani et al 1996).

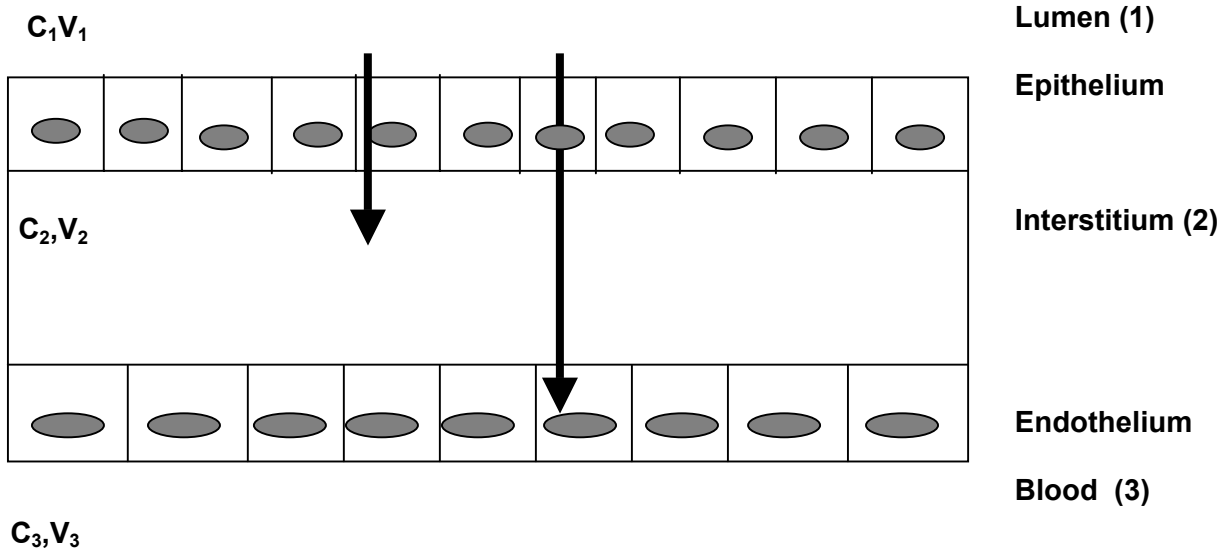



**Fig.3**

An illustration of the main barriers (endothelium and epithelium) of particle translocation in the lung and the two main pathways of translocation (i.e. paracellular and transcellular).

As illustrated in Fig. 2 oxidative stress, inflammation and the possible local release of vasoactive mediators may be induced by particle inhalation and deposition and these processes may again indirectly affect translocation of the ultrafine fraction.

### 1.2.4 Epithelial lining and lung permeability

The lung epithelium is in direct contact with the environment that makes the airspace epithelial surface of the lung vulnerable to the effects of oxidative stress (MacNee 2001) .Ultrafine particles are able to penetrate deeply into the respiratory tract and due to their large surface area than the particles of large size, the cause a greater inflammatory response

in the lung (MacNee and Donaldson 2000) The chronical exposure to several types of mineral dust particles might induces inflammatory reaction in the lung (Doelman et al 1990). Particles might activate alveolar macrophages and prime leukocytes that can lead to enhanced release of reactive oxygen species. Particles might also contain radicals. These reactive oxygen species may lead to tissue damage. Increased epithelial permeability is present in chronic smokers' whit changes in airspace antioxidants e.g glutathione. The oxidant burden in the lung is further increase with the release of activated neutrophils and macrophages in the alveolar spaces (MacNee 2001).

### 1.2.5 Endothelial layer lung permeability

One third of the endothelial cells in the body are in the pulmonary vasculature and thus receive all the cardiac blood output and therefore represents a target organ (Christafidou-Solomidou et al, 2000). The cut-off size of tight junctions between alveolar type I cells is 0.6 nm but the endothelial junctions allow passage of larger molecules around 4-6 nm (Agu et al 2001). Physical barriers like the mucus (1-10 μm thick) linking the pulmonary epithelium and the surfactant that lines the alveoli (0.1-0.2 μm thick) may hinder pulmonary absorption (Agu et al 2001). Although others have suggested that there is an essential role of surfactant in particle translocation (Geiser et al, 2003). Any particle that reaches the alveoli will be either degraded by proteases or removed by alveolar macrophages. The pulmonary macrophages secrete also peroxidases, inflammatory and immunomodulatory mediators as well as other molecules as part of the host defence mechanism. However, ultrafine particles are able to disperse in the fluid of epithelial lining so that the lung receives a dose of singlet particles (Donaldson et al, 2001). Except from the organs like the liver, adrenal and bone marrow sinusoids where the endothelium has rather large pores, the endothelium constitutes a selective barrier between blood and tissue (Gumbleton , 2001). Inflammatory mediators are thought to induce vascular leakage or causing gaps between endothelial cells that result in crossing of blood elements or plasma proteins across the endothelium. This increase endothelial permeability helps and facilitates blood elements to sites of injury (Tiruppathi et al, 2002). The activation of endothelial cells by thrombin plays an important role in the pathogenesis of vascular injury and tissue inflammation. Thrombin increases causing vascular permeability and tissue water content in the lungs (Lum et al, 1994). It believes that thrombin mediates this effect by activation of G-protein coupled proteinase activated receptor-1 (PAR-1) expressed in the endothelial cell surface. The increase of intracellular Ca++ signalling is critical in the mechanism of increased endothelial permeability after activation of PAR-1 (Sandoval et al, 2001). The thrombin increase in permeability was reduced after blocking Ca++ influx in the endothelial cells (Sandoval et al, 2001). Another

inflammatory mediator is TNF-alpha and its effects have been clearly shown in conditions as inflammation and sepsis in which the endothelial cells are the first target of this cytokine (Tiruppathi et al, 2002). Increase ROS production in endothelial cells may interfere with crucial endothelial functions such as nitric oxide and COX pathways (Kojda et al, 1999).

### 1.3 Models to study systemic effects of particles

Current epidemiological data do not allow drawing conclusions on the mechanism of particle to cause systemic effects, although interesting efforts have been published (Pope, 2004). Current animal models for human cardiopulmonary research are limited and mostly use compromised animals either by genetic, pharmacological or surgical manipulation of the cardiopulmonary system. These models have both strengths as well as limitations as to how they really mimic the essential features of human cardiopulmonary diseases. Various animal models including pulmonary hypertension, bronchitis, asthma and cardiovascular disease, and emphysema have used for these studies and are summarised in Table 3. Some studies suggested that certain individuals may be at higher risk for adverse effects of PM showed a greater susceptibility to air pollution particulate matter (Costa and Dreher, Dockery et al, 1993, Schwartz 1994). Host susceptibility however appears to involve multiple genetic and environmental factors in humans and this is poorly understood. Considering the objectives of our study, which concern the dissection of direct and indirect mechanisms in particle induced effects, it is an obvious choice not to use an in vivo model, since one cannot discriminate between these pathways in vivo. For instance, an inflammatory response will always be able to modify a direct mechanism such as particle particle translocation. Therefore we choose for two perfusion models using the isolated perfused lung and on the other hand the retrograde coronary perfused heart, the Langendorff model. The advantage of these models is that they retained structural integrity, but also excluded control by central innervation mechanisms and infux of inflammatory cells.

**Table 3.**

An non-comprehensive overview of animal models that has been used to study the systemic effects of PM and/or model particles. A lot of mouse work (ApO-E mice was published in 2005, e.g. Inhalation Tox)

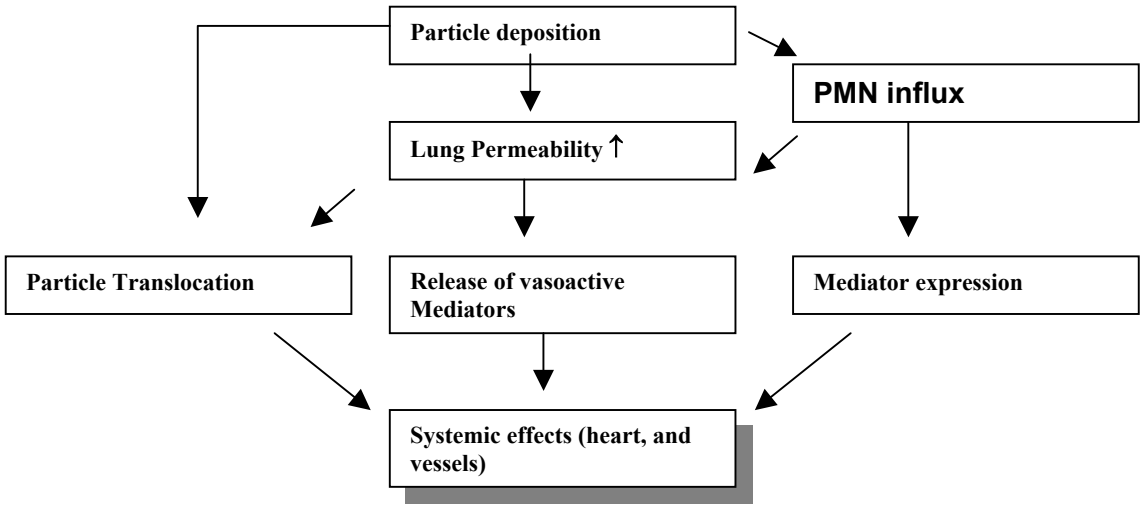
| Species | Model/Treatment | Outcome | Authors |
|---|---|---|---|
| SD rats (male),7-8 wks | ROFA (It) | Inhibition of in vivo arterial dilatation | Nurkeiwicz et al, 2004 |
| F344 aged rats (> 23 months) | UF carbon particles (LPS injected) | UFP did not induce lung inflammation, but decrease blood PMN's and affect TAT complex and fibrinogen levels | Elder et al, 2004 |
| SHR , aged rats (11-14 months | UF carbon particles (LPS injected) | | Elder et al, 2004 |
| Sprague Dawley (250-300g ) Monocrotaline (MCT) | ROFA (It and Nose ) | Pulmonary lesions, thickening of alveolar wall | Kodavanti et al, 1999 |
| Male F344 rats (18 months old | CAPS, SO2 and air .Nose only exposure | (Arrhythmias) Increases in frequency of irregular and delayed beats for CAPS | Nadziejko et al, 2004 |
| AKR/J strain Inbred mice (Senescent) | Carbon Black | Heart Rate and Heart Rate Variability | Tankersley et al, 2004 |
| Spontaneously Hypertensive rats | Low metal combustion –derived particulate matter | ECG, Heart Rate, Blood Pressure, Core Temperature | Wichers et al, 2004 |
| Spontaneously Hypertensive rats | Low metal combustion –derived particulate matter | Biochemical indices of pulmonary inflammation and injury | Wichers et al, 2004 (b) |
| Spontaneous Hypertensive rats | Concentrated PM2.5 | Heart Rate and Blood Pressure | Chang et al, 2004 |

**Abbreviations :** SD, Sprague Dawley ; F344, Fisher ; SHR, Spontaneous Hypertensive rats; ROFA, Residual oil fly ash ; CAPS, concentrated air particulates

### 1.5 Aims and outline of this research

The primary aim of the studies in this thesis was to investigate if the translocation of ultrafine particles is a mechanism that can explain cardiovascular effects, as observed in epidemiological studies. We therefore evaluated the translocation of model nanoparticles ($^{192}$Ir-aersol, 18 nm) through the lung in an isolated perfused lung system (Chapters 2 and 3). In addition we used in vivo administration of ambient PM in a compromised animal model to evaluate its effects on cardiovascular function using Langendorff isolated heart perfusion (Chapter 4). The second aim was to investigate the role of lung permeability in particle translocation and the effect of lung inflammation on these processes (Chapter 2 and 3). For this purpose we also used the isolated lung perfusion model, with treatments to modify permeability (histamine) and/or lung inflammation (neutrophils). Both model particles ($^{192}$Ir-aersol, 18 nm) and ambient PM (EHC-93) were used in our studies. The conceptual framework for this thesis is illustrated in figure 5 and indicates that particles may translocate through lung epithelium and affect cardiovascular function directly. On the other hand, particles may also induce inflammation, which affects lung permeability to both particles and vasoactive mediators.

Fig 5. Schematical presentation of mechanisms and their sequence, suggested to play a role in particle induced systemic effects, centrally mediated through lung permeability. The scheme indicates that particles may translocate through lung epithelium and affect cardiovascular function directly. On the other hand, particles may also induce inflammation, which affects lung permeability to both particles and vasoactive mediators.



### 1.5.1 Isolated perfused lung model

The isolated perfused rat lung model (IPL) has been used over the past 80 years to study hemodynamic parameters (Fisher,1985), hypoxic vasoconstriction (Emery et al, 2003, Chlopicki et al, 2002), pulmonary action of histamine or the arachidonic acid metabolites (Russel et al, 1994), and the effect of environmental pollutants (Bond & Mauderly, 1984). The IPL model offers many advantages over other methods to study pulmonary metabolism and effects of exogenous agents. The IPL is much less complicated than the whole animal while preserving most of the integrity of the organ. Unlike experiments in intact animals, perfusion experiments allow to retain control over and monitoring of parameters such as perfusion pressure such as tidal volume, pulmonary compliance, pulmonary resistance, vascular resistance, vascular compliance, weight gain, filtration coefficient and blood gases from each single experiment (Uhlig et al, 1994). With regard to inflammation a major advantage is that a cell free perfusate allows controlling inflammation and avoiding bias by particle uptake in leukocytes and other cells. The IPL can be operated in various ways, including constant flow perfusion (CFP) which mimics the *in vivo* situation more closely than the constant pressure perfusion (CPP). In addition, two different ventilation modes can be used, i.e. negative or positive pressure ventilation. Negative pressure ventilation (NPV) has been considered preferable because it closely mimics the *in vivo* conditions (Uhlig and Heiny, 1995). NPV produces less oedema formation and higher cardiac output than PPV. On the other hand, the IPL also has limitations, since it can only be used for a limited duration. The lung mechanics start to deteriorate after some time. Another limitation is that the lung is deprived of nervous regulation and lymph drainage which can affect lung function. We considered the IPL to be suitable for our experiments on particle translocation, since it has been used previously to evaluate the uptake of various pharmaceuticals, including aerosol formulations (Niven and Byron 1988), surfactant absorption enhancers (Niven and Byron, 1990) and the absorption of synthetic polypeptides (Niven et al 1990). These studies were performed in the context of macromolecular delivery to the systemic circulation.

### 1.5.2 The Langendorff model

The isolated perfused Langendorff heart is a suitable model to investigate effects of ambient particles on the heart. The isolated heart from small mammals provides a highly reproducible preparation that can be studied quickly and in large numbers (Kreutzer *et al*, 2004, *Vork et al*, 1993). This preparation is relatively cheap and allows measuring or controlling a broad spectrum of biochemical, physiological, morphological and pharmacological indices. Advantages of the isolated perfused heart model include that measurements can be made in

the absence of the confounding effects of other organs, the systemic circulation and a host of peripheral complications such as circulating neurohormonal factors (Schunkert *et al*, 1995). Another advantage of the isolated heart preparation is the allowance of experiments to be continued in the face of events e.g. infarction-induced loss of pump function (Schwartz et al, 1993) , cardiac arrest ( Ejike *et al*, 2004) or arrhythmias (Valentin *et al*, 2004) , which would normally jeopardise the survival of an in vivo experiment.

The isolated heart can be maintained for several hours but gradually deteriorates with time. The rat heart is by far the best characterised and is also the system most frequently used for more complex perfusion preparations such as working and blood perfused hearts. An additional advantage of the rat heart is that it's easy to handle and its size enables intraventricular recordings. One limitation of the rat heart is the very short action potential duration which can limit its value in arrhythmogenesis and anti-arrhytmmic drugs studies (Hearse and Sutherland, 2000). Isolated perfused heart preparations are largely based on adaptions of that originally described by Langendorff and the more complex working preparation described by Neely (Neely et al, 1967). The Langendorff heart preparation involves the cannulation of the aorta which is attached to the reservior containing oxygenated perfusion fluid. The fluid is delivered in a retrograde direction in the aorta either at a constant flow rate by an infusion or roller pump or using a constant hydrostatic pressure usually in the range of 60-100mmHg. In both these instances, the aortic valves are forced to shut and the perfusion fluid is directed into the coronary ostia thereby perfusing the entire ventricular mass of the heart, draining into the right atrium via the coronary sinus. Once the heart is securely attached to the cannula and coronary perfusion initiated, contractile function and regular heart rhythm will start. Usually a 10 minute stabilisation period is needed before contractile function measurements are started.

### 1.5.3. Outline

The above ex-vivo perfusion models were applied to study the central aims of this thesis, i.e. to evaluate the importance of particle translocation and the relevance of lung inflammation or permeability in this process. In Chapters 2 and 3 we describe the outcomes of studies done with model nanoparticles in isolated perfusion lung in different set-ups. While in Chapter 2 we used inhalation of NP and negative pressure ventilation of IPL, we used intratracheal instillation and positive pressure ventilation in Chapter 3. In both set-ups the same model particles ($^{192}$Ir-aersol, 18 nm) were used with experimental set-ups designed to modify endothelial permeability (histamine), epithelial permeability (hydrogen peroxide) and inflammation (PMN in the perfusate). Subsequently, in chapter 4 we studied the effects of

ambient particles (EHC-93) on cardiovascular function, and compared this to endotoxin as a reference to induce the inflammatory response only. Both particles and LPS were instilled *in vivo* and heart performance was measured at different time points after instillation *ex-vivo* after subjection to ischaemic insult, to mimic myocardial infarction *in vivo*. Additional studies on plasma biomarkers and isolated cardiomyocytes were performed to evaluate the importance of transition metals in the effects observed in the heart perfusions.

**Chapter 2**

# The influence of hydrogen peroxide and histamine on lung permeability and translocation of iridium nanoparticles in the isolated perfused rat lung

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# ABSTRACT

**Background.** Translocation of ultrafine particles (UFP) into the blood that returns from the lungs to the heart has been forwarded as a mechanism for particle-induced cardiovascular effects. The objective of this study was to evaluate the role of the endothelial barrier in the translocation of inhaled UFP from the lung into circulation. **Methods.** The isolated perfused rat lung (IPRL) was used under negative pressure ventilation, and radioactive iridium particles (18 nm, CMD, $^{192}$Ir-UFP) were inhaled during 60 minutes to achieve a lung burden of 100 - 200 µg. Particle inhalation was done under following treatments: i) control perfusion, ii) histamine (1 µM in perfusate, iii) luminal histamine instillation (1 mM), and iv) luminal instillation of $H_2O_2$. Particle translocation to the perfusate was assessed by the radioactivity of $^{192}$Ir isotope. Lung permeability by the use of $Tc^{99m}$-labeled diethylene triamine pentaacetic acid (DTPA). In addition to light microscopic morphological evaluation of fixed lungs, alkaline phosphatase (AKP) and angiotensin converting enzyme (ACE) in perfusate were measured to assess epithelial and endothelial integrity. **Results.** Particle distribution in the lung was homogenous and similar to in vivo conditions. No translocation of Ir particles at negative pressure inhalation was detected in control IPL, but lungs pretreated with histamine (1 µM) in the perfusate or with luminal $H_2O_2$ (0.5 mM) showed small amounts of radioactivity (2- 3 % dose) in the single pass perfusate starting at 60 min of perfusion. Although the kinetics of particle translocation were different from permeability for $^{99m}$Tc-DTPA, the pretreatments ($H_2O_2$, vascular histamine) caused similar changes in the translocation of particles and soluble mediator. Increased translocation through epithelium and endothelium with a lag time of one hour occurred in the absence of epithelial and endothelial damage. **Conclusions:** Permeability of the lung barrier to UFP or nanoparticles is controlled both at the epithelial and endothelial level. Conditions that affect this barrier function such as inflammation may affect translocation of NP.



**Keywords** : endothelium, translocation, ultrafine particles, isolated perfused lung, permeability

## Introduction

Epidemiological studies have demonstrated an increased morbidity and mortality by particulate air pollution (Dockery et al, 1993 ; Pope at al, 2002). The highest relative risk for mortality and hospital admissions were observed in subjects with existing pulmonary disease including asthma and COPD (Dockery et al, 1993 ; Pope at al, 2002). The exact mechanism by which PM can adversely affect humans remains unknown, but several hypotheses have been forwarded. These include that PM causes pulmonary inflammation causing release of factors that influence blood coagulation ( Seaton et al, 1995), reduced lung function (Hoek et al, 1998), increased blood plasma viscosity (Peters et al, 1997), reduced heart rate variability ( Gold et al, 2000; Pope et al, 1999) and destabilisation of atheromatous plagues ( Suwa et al, 2002). Some of these effects are attributed to translocated nanoparticles based on their potential effects on vascular function (Bagate et al, 2004a ; Nurkiewicz et al, 2004), blood coagulation ( Nemmar et al, 2002), mitochondrial function Li et al, 2003) and Ca-flow (Stone et al, 2000 ; Oortgiesen et al, 2000).

Nanoparticles have been shown to translocate from lung to the circulation (Kreyling et al, 2002 ; Nemmar et al, 2002 ; Nemmar et al, Nemmar et al, 2002 ; Oberdorster et al, 2002), but most of the inhaled dose remains in the lung interstitium (Ferin et al, 1992) even up to several years (Borm et al, 2004). Therefore it seems that not the epithelial but the endothelial barrier is more important in prevention of translocation to the blood. Enhanced lung permeability has been measured by increased Clara-cell protein in blood (Bernard et al, 1997) or enhanced DTPA clearance in the lung (Royston et al, 1990) after ozone and hyperoxia. Recent work in rabbit isolated perfused lungs shows that nanoparticles themselves can influence microvascular permeability measured by weight gain after occlusion (Hamoir et al, 2003). However, since the mechanisms of nanoparticle transport on a sub-cellular level are unknown it remains to be determined whether the above indices of lung permeability are related to translocation of nanoparticles. Particles may also cause the release of vasoactive mediators such as histamine, which was shown to be increased in plasma of hamster after instillation of diesel exhaust particles (Nemmar et al, 2004). Histamine is well known to induce vascular permeability through its action on endothelial $H_1$-receptor (Abbot 2000). Finally, by oxidative stress mechanisms ambient and nanoparticles can cause activation of lung alveolar macrophages and epithelial cells that result in the production of pro-inflammatory cytokines such as TNF and Il-1 in humans (Schaumann et al, 2004) and rat models (Dick et al, 2003) that are typically associated with increased lung permeability (Mullin et al, 1990 ; Lee et al, 2000).

The objective of this study was to assess nanoparticle translocation in relation to permeability changes for small molecules and integrity of epithelial and endothelial monolayers. In order to manipulate permeability in the absence of neutrophil recruitment and activation we used an isolated perfused rat lung. Several treatments to modify lung permeability in-vitro were applied including oxidative stress by instillation of hydrogen peroxide and endothelial permeability by histamine in the perfusate. These treatments were selected for their relevance to conditions of patients with pulmonary or systemic complications. Particle translocation was assessed by the inherent radioactivity of 18 nm size iridium nanoparticles ($^{192}$Ir-UFP).

## MATERIALS & METHODS

### Animals and surgical procedure

Adult, healthy, male Wistar-Kyoto rats (WKY/Kyo@Rj rats, Janvier, France) (200- 250 g) were housed in pairs in a humidity (55% relative humidity) and temperature ( $22^0$C ) controlled room. They were maintained on a 12-h day/night cycle. Rats were allowed to acclimate to the facility for a minimum of 10 days prior to use. When the experiments were performed rats were more than 17 weeks of age. The studies were conducted under federal guidelines for the use and care of laboratory animals and were approved by the Oberbayern Government and by the GSF Institutional Animal Care and Use Committee. Surgical procedure for lung isolations was done according the method of Uhlig and Wollin (1994). Briefly, rats were anaesthetized intraperitoneally with 80 mg/kg ketamin. Deep anesthesia was characterized by a lack of response to toe pinching. Heparin (500 IU) was injected via the tail vein. A midline incision was made from the pelvic region to the neck of the rat. With the ventilator operating, the trachea was cannulated using a rigid catheter and the catheter was attached to the ventilator. Therefore lung were ventilated from the start of the whole procedure. The animals were exsanguinated opening the aorta abdominalis after deep intraperitoneal anesthesia with ketamine (100 mg/100 g body weight) and xylazine (0.5 mg/100 g body weight). After anesthesia a longitudinal ventral incision was made to open the thoracic and abdominal cavity and it was held open using clamps. The thymus was removed and the apex of the heart was cut off to introduce a cannula into the pulmonary artery. A slight perfusion flow of around 1 ml/min was maintained before inserting cannula. Care was taken not to introduce any air bubbles into the pulmonary artery. The left atrium was cannulated by advancing the venous cannula through the mitral valve. A ligature was placed around the heart to keep both cannula's in place. The aortic cannula was then attached to the lining fed through the Perspex lid of the 500 mL negative-pressure-chamber. After the Perspex lid was fully mounted on the chamber negative pressure ventilation started. The respiratory settings during the negative pressure ventilation were 65 breaths per min. Regular sighs were introduced (hyperventilation) to improve function of lungs.

### Isolated lung perfusion

The IPL-4401 Isolated lung ventilation perfusion system (FMI GmbH Oberbach) was used for our study. Additional negative pressure chamber has been constructed by GSF –National Research Centre for Environment and Health. The system in brief consists of a small animal ventilator, jacketed upper media reservoir, negative-pressure-chamber holding the heart-

lung-bloc, perfusion lines, peristaltic pump and pulmonary artery pressure transducer. The computer software (FMI GmbH Oberbach) was operated by a 386 personal computer and allowed constant monitoring of pulmonary blood pressure. The upper media reservoir, ventilation chamber and perfusion lines were held at 37 $^{0}$C by a re-circulating water-bath. The perfusion medium was selected based on its extensive use in isolated organ perfusion and consisted of a modified Krebs-Ringer –bicarbonate buffer. Krebs-Ringer composition was as follows (mM): NaCl 118 ; KCl 5.9 ; $CaCl_2$ 2.5 ; $MgSO_4$ 1.2 ; $NaH_2PO_4$ 1.2 ; $NaHCO_3$ 24.9 ; glucose 11.1 , pH was adjusted at 7.4. It was then mixed at a ratio 1:1 with Haemacell solution (Hoechst Marion Roussel). The buffer was pre-warmed and gassed with 95% $O_2$ and 5% $CO_2$ at a rate low enough to prevent excessive frothing of the medium. The medium flowing through the system passed through a bubble trap prior to reaching the lungs and the buffer pH was continuously monitored throughout the experiment. Respiratory rate was set at 65 breaths per min. Lungs were inflated at a maximum negative pressure of -1.5 kPa in the chamber. Stroke volume was set at 10 ml to achieve a tidal volume of usually 3-4 ml (because of the altered compliance of the lungs). The lungs were expanded (sighed) every 4 minutes applying a negative pressure of -2.5 kPa to the chamber. Optimal perfusion settings included perfusion rate of 5 ml/min and a medium pH of 7.4. Perfusion pressure was not constant but was kept between 10 and 14 kPa.

**Particle generation and exposure**

Aerosols of ultrafine iridium particles (Ir-UFP) radio labeled with $^{192}$Ir were produced with a spark generator as described previously (Kreyling et al, 2002). Size distribution and number concentration were monitored continuously by a differential mobility particle sizer (DMPS 3070, TSA instruments) and a condensation particle counter (CPC 3022A, TSI Instruments). The size distribution of the $^{192}$Ir-UFP was aimed to a count median diameter of 17-20 nm (geometric standard deviation 1.6) at a particle concentration of $10^7$ cm$^{-3}$ aiming for a tidal volume of 3-4 cm³ at a frequency of 65 /min. The estimated dose under these conditions is 180 µg/hour. A schematical description of the experimental system is shown in Figure 1

**Experimental design**

Following a 15 minute period of equilibration, during which the lungs were already ventilated and perfused, the experiment started by the inhalation of freshly produced $^{192}$Ir-UFP from the aerosol line. Intratracheal instillation of $^{99m}$Tc-DTPA or other instillations were performed at this starting time point. The perfusate was collected continuously and sampled at 15-min time intervals (Figure. 2). The following treatments were investigated,

group I: control group, only $^{192}$Ir-UFP inhalation for 120 min, ;
group II: instillation of 50-100 µL $^{99m}$Tc-DTPA, 500 µl $H_2O_2$ bolus (10mM), $^{192}$Ir-UFP aerosol inhalation for 120min;
group III: instillation of 50-100 µL $^{99m}$Tc-DTPA, histamine continuously perused during the next 2 hours at concentration 10 µM, $^{192}$Ir-UFP aerosol inhalation for 120 min;
group IV: instillation of 50-100 µL $^{99m}$Tc-DTPA and 500µl histamine bolus instillation at a concentration of 10 mM, $^{192}$Ir-UFP aerosol inhalation for 120 min;
group V: instillation of 50-100 µL $^{99m}$Tc-DTPA.

**Evaluation of $^{192}$Ir-UFP translocation**

The perfusate samples as well as the heart-lung-blocs were analysed for $^{192}$Ir-UFP activity in a shielded 1-L-well-type gamma-spectrometer. Analysis of $^{192}$Ir activity was performed in those samples studied for $^{99m}$Tc-DTPA permeability when the $^{99m}$Tc activity had decayed – see below. Activity measurements of both isotopes were decay and background corrected. $^{192}$Ir activity in the perfusate samples were given as a fraction of the total activity found in the perfusate and the heart-lung-bloc.

**Evaluation of lung permeability**

Technetium-99$^{m}$ labeled DTPA ($^{99m}$Tc-DTPA; DRN 4362 TechneScan-DTPA , Malinckrodt Medical BV, The Netherlands) was used to evaluate lung permeability. The lyophilised DTPA powder was dissolved in 10 ml sterile $^{99m}$Tc activity containing saline, which was eluted from the $^{99m}$Tc generator. The solutions were then allowed to equilibrate for 15 minutes at room temperature. The volume instilled in the trachea was 50-100 µL at a DTPA concentration of 120-250 µg. and a $^{99m}$Tc activity of 5-10 MBq. The $^{99m}$Tc-DTPA permeability was studied measuring the activity in the heart-lung-bloc and the perfusate samples. The $^{99m}$Tc radioactivity was also analysed in the shielded 1-L-well-type gamma-spectrometer at the appropriate photo peak of $^{99m}$Tc. Since the $^{99m}$Tc activity was chosen to be at least an order of magnitude higher than the $^{192}$Ir deposition in the lungs, interference of Compton rays in the $^{99m}$Tc window originating from $^{192}$Ir was negligible. Permeated $^{99m}$Tc activity in the perfusate samples was given as a cumulative fraction of the total instilled activity recovered in the perfusate and the heart-lung-bloc.

**Tissue preparation and microscopy**

Immediately after the termination of the lung perfusion the radioactive particles treated lungs were air dried with room air at a pressure 3.5 kPa and subsequent imaging for particle distribution. For histopathology only lungs treated with non-radioactive iridium particles were used. After the experiment, the trachea and pulmonary vein of the IPL were perfused with 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2, 340 mOsm) at 25 cm fixative pressure. Post-fixation of the lungs was done by immersion of the whole lung in the same fixation solution for 2 hours at room temperature. t 25 cm fixative pressure. Two slices from left- and right caudal lobes of each animal were embedded in paraffin and 5 µm thick sections were stained with hematoxylin and eosin. Small portions of the left lobe of an sub-group of 7 animals were embedded in Epon ®, and semithin sections (1 µm) were stained by toluidine blue.

**Biochemical analysis of the perfusate**

Perfusate samples were analysed for histamine with an ELISA kit from IBL-Hamburg (reference no. RE 59221). The detection limit of the kit was 0,3 ng/ml when using plasma. Angiotensin converting enzyme (ACE) was measured according the kinetic method of Maguire and Price (Maguire and Price 1985) using standards from Bühlmann Laboratories AG, Switzerland (Reference KK-ACK), Protein determination was done according the Bicinchonic acid (BCA) protein assay (Smith et al, 1985). Clara-cell protein was measured in perfusate using a sensitive latex immunoassay (Bernard et al, 1997) with a detection limit 1 µg/l perfusate. Alkaline Phosphatase (ALP) determination was done with KIT manufactured by Diasys Diagnostics GmbH Germany Cat no: 104019990314. Samples were measured (Beckman DU 640 spectrophotometer) at 25 °C at wavelength 405 nm. The increase of the extinction was measured each minute for 3 min and enzyme activity was measured as the difference in extinction divided by the minutes multiply a constant factor 2754 (Fischbach and Zawta, 1992).

**Statistical Analysis**

Results are expressed as means ± SD, and/or as individual experiments (Fig5).Differences between treatments were tested for statistical significance by Mann Whitney-test. A value of $P < 0.05$ was considered significant. All statistics were run with SPSS for Windows XP.

## RESULTS

### Particle distribution and deposition

The first set of experiments measured the distribution of $^{192}$Ir -UFP related radioactivity in the lungs. Deterioration of lung performance was noted based on increasing frequency of inflation to maintain tidal volume, but could not be quantified during negative pressure perfusion in the experimental set-up due to maintain a closed system for radiation protection safety reasons. In a positive pressure using the same equipment, tidal volume, respiration pressure and weight did not change over a 2-hour perfusion period. The particle size distribution in the inhaled aerosol was well reproducible and the count median diameter (CMD) ranged between 16 and 18 nm of particle diameter (Fig 3). Geometric standard deviation (GSD) always was 1.6. Deposition of particles in isolated perfused lungs was compared to animals exposed parallel to the same aerosol and showed similar homogenous distribution, with somewhat lower deposition (data not shown).

### Translocation of ultrafine particles after modified permeability

In a large set of perfusions in control lungs no translocation of $^{192}$Ir-UFP particles was noted and the variance between different perfusions is small (< 5 %) as shown in Fig. 4A. Then several treatments were applied to investigate the role of epithelial and endothelial permeability on particle translocation. First, hyperinflation to double tidal volume every minute was applied but did not lead to increased translocation of nanoparticles (data not shown). An initial bolus injection of $H_2O_2$ into the trachea of the IPL to reach a final concentration of 0.5 mM, caused particle translocation to start at 60 min after onset of the inhalation of radioactive aerosol. A significant difference ($P<0.05$, Mann-Whitney U-test) in particle-related radioactivity in the perfusate was observed between control and $H_2O_2$ group at 90, 105 and 120 minutes after onset of inhalation (Fig. 4A). At other time-points beyond 60 minutes the differences to untreated lungs were of borderline significance ($P < 0,1$; Mann Whitney-test). The variance between perfusions upon this treatment in Fig 4A was much higher than in control perfusions. However, individual presentation of the experiments of $H_2O_2$ pretreated lungs (Figure 5A) show a similar trend in all perfusions. Increased radioactivity in perfusate was only detected beyond 60 minutes of perfusion. A similar translocation versus time profile was observed in lungs upon presence of 1 µM histamine in the vascular perfusion fluid (Fig 4A). However, here statistical significance in this condition versus control lungs was only attained after 120 minutes of perfusion (Fig 4A), which is best explained by the individual experiments shown in Fig 5B. On the other hand, in the lungs

treated with a histamine bolus injection no $^{192}$Ir-UFP radioactivity was detected in the perfusate.

Interestingly, the kinetics of translocation of DTPA (Fig. 4B) and Ir-UFP are very different. Whereas Ir-UFP only starts to increase in perfusate after 60 min of inhalation, DTPA is measured in perfusate within a few minutes after intratracheal instillation. On the other hand the effects of $H_2O_2$ and vascular histamine on particle translocation are also reflected in the DTPA –clearance (Fig. 4B). Although not significant, both treatments caused trends of a higher rate of translocation of DTPA one hour after administration, which is also observed for translocation of $^{192}$Ir-UFP. The histamine bolus injection, with a final target concentration in the lumen of 0.5 mM caused a considerable slowing-down of DTPA permeability (Fig 4B) and no observed effects on $^{192}$Ir-UFP translocation.

**Biomarkers of epithelial and endothelial damage**

Alkaline phosphatase (ALP) was measured in control and pre-treated lungs as a marker of type II cell damage. No significant differences in ALP activity (15-135 minutes) were observed between perfusate of the control IPL and $H_2O_2$ pre-treated lungs after exposure to $^{192}$Ir-UFP ( Fig 6A). In the IPL perfused with vascular histamine a significantly lower activity of ALP was seen at 15 and 30 minutes in comparison to lung perfusions that only received $^{192}$Ir-UFP by inhalation. At all later time points ALP showed no difference to control lungs (Fig 6A). The histamine bolus group did also not differ from control group. To evaluate endothelial damage, angiotensin converting enzyme (ACE) was measured in the lung perfusate (Figure 6B). No significant differences were observed in ACE activity between the control group and isolated lungs treated with $H_2O_2$, histamine in perfusate or histamine delivered as a bolus in the trachea (ANOVA, post-hoc Tukey and Mann Whitney-test). To check whether the $H_2O_2$ effect was mediated by histamine release we measured histamine in the perfusate but did not detect significant differences to histamine levels in control perfusions (data not shown). Also a measure of total protein for lung permeability did not detect differences between the different treatments used in these experiments.

**Morphology of lungs**

The outcomes of the histo-pathological analyses are summarised in Table 1 and illustrated in Fig. 7. Overall there is quite extensive damage at the end of the perfusion experiments, but there are not many differences between the different treatments. Sub-epithelial round cell infiltration, interstitial dilation along with moderate to severe oedema was present in all the groups. Occasionally alveolar dilation, alveolar inflammation and fluid in the alveolar lumen

were detected. The in vitro perfusion procedure might be responsible for the perivascular and peribronchial dilation in all the lungs. $H_2O_2$ might be directly responsible for the epithelial damage of the proximal bronchi in the $H_2O_2$ instilled group.

## DISCUSSION

The purpose of our study was to evaluate the role of epithelial and endothelial barrier in the translocation of ultrafine particles across the lung into the systemic circulation using the isolated perfused lung model. Translocation of Iridium (Ir) particles was monitored by radioactivity of the particles themselves, and not by any attached radioactive label. No translocation of $^{192}$Ir-UFP (17-20 nm) was detected in isolated perfused rat lungs. However lungs pre-treated *in-situ* with histamine on the endothelial side (1 µM) or $H_2O_2$ (0.5 mM) in the alveolar lumen showed small amounts of radioactivity in the single pass perfusate after a lag-time of 60 min. Although kinetics of DTPA and particle translocation was different the *in-situ* treatments histamine and $H_2O_2$ caused unidirectional in both processes, in the absence of biochemical evidence for epithelial and endothelial damage.

In this study we applied several *ex-vivo* treatments of the isolated lungs with either $H_2O_2$ or histamine. Using $H_2O_2$ we anticipated inducing an oxidative stress, which is also caused by PM inhalation in the lung both by direct radical formation by PM constituents and indirectly by recruited inflammatory cells (review: Knaapen et al, 2004). Oxidative stress has been forwarded as a central hypothetical mechanism in the adverse effects of PM, including ultrafine particles Donaldson et al, 2003). Actually, the oxidative capacity of PM was shown by us to be a predictor of bronchial inflammatory response to PM after installation in normal human volunteers (Schaumann et al, 2004). Earlier on Rhaman et al, (2001) forwarded that oxidative stress and depletion of GSH can affect lung permeability allowing for greater particle passage via lung epithelium into the interstitium. This concept is supported by our data using a high concentration of $H_2O_2$ (5 mM) by bolus injection into the lung, to reach a final concentration of 0.5 mM. In fact, lung-lining fluid of COPD patients has been shown to contain levels of $H_2O_2$ up to 5 µM (De Benedetto et al, 2000). Although this model does certainly not meet all conditions of an inflammatory response, similar models have been applied in other ex-vivo permeability studies (Habib and Clements, 1995 ; Hulsmann et al, 1996). In isolated perfused rat lungs, a low concentration of $H_2O_2$ (0.25 mM) in the perfusate was shown to increase capillary permeability in the absence of lipid peroxidation (Habib and Clements, 1995). A short-term treatment with $H_2O_2$ (100mM) on the epithelium of human airway tubes caused a six-fold increase in translocation of $^{111}$In-DTPA, which was explained by the opening of paracellular pathways (Hulsmann et al, 1996). In our study, we assume that a final luminal concentration of 0.5 mM $H_2O_2$ is reached and we found an increased translocation of both $^{192}$Ir-UFP as well as a trend of increased translocation of $^{99m}$Tc-DTPA after a lag-time of about 60 min. However, no temporal relationship between both markers of translocation was seen, which suggest that they operate through different routes.

More data on the translocation route of $^{192}$Ir-UFP in the lungs are given by the results obtained with histamine administered both on the luminal side and through the microvasculature. These data show that histamine at very low levels in the perfusate (1 µM) caused an increased translocation of particles as well as $^{99m}$Tc-DTPA-permeation after a lag-time of about 60 min (Fig 4). On the other hand luminal administration of histamine (0.5 mM) did not increase $^{192}$Ir-UFP translocation and actually showed a slightly reduced $^{99m}$Tc-DTPA permeation. Histamine is a very potent vasoactive and bronchial mediator that has been shown to be involved in both local and systemic effects of diesel particles (Nemmar et al, 2004). First, upon mast cell degranulation histamine is the major mediator of bronchial constriction as observed in allergic airway response (Nadel and Barnes, 1984). This constriction probably also explains our reduction in $^{99m}$Tc-DTPA clearance after a histamine bolus injection into the trachea. The approach using vascular histamine is relevant because histamine has been shown to increase after instillation of particles in isolated tracheally perfused rabbit lung *ex-vivo* (Nemmar et al, 1999), hamsters *in vivo* (Nemmar et al, 2003) and healthy human volunteers (Salvi et al, 1999). We assume that histamine at low concentrations ($10^{-6}$ M) in our system increases endothelial permeability (Leach et al, 1995) and allows an increase in intercellular transport of $^{192}$Ir-UFP located in the interstitium through the endothelium into the perfusate. A similar effect of $10^{-4}$ M vascular histamine was found recently in isolated perfused rabbit lungs (Nemmar et al, 2005). In the latter study latex particles (24- 190 nm) translocated from the vascular compartment into the lumen, as observed by subsequent bronchoalveolar lavage. The amount of reverse translocation was about 2.5 % of administered dose within 2 hrs of perfusion. No translocation of latex particles with different size (24-190 nm) and surface chemistry (carboxylate versus amine) and charge were seen under normal physiological conditions in the rabbit lungs. In our studies 18 nm iridium particles also did not translocate through rat lung barriers, although we used negative pressure ventilation which caused considerable damage to the lung tissue.

Taken together these findings suggest that the translocation of ultrafine particles in the lung occur through different routes. Among different uptake routes we can discriminate transcytosis and para(inter)-cellular transport. Hermans et al (1999) stated that radiolabelled tracers such as $^{99m}$Tc-labelled DTPA permeate the epithelial barrier by passing through intercellular junctions. Since kinetics of DTPA and $^{192}$Ir-UFP translocation in untreated lungs is so different, we suggest that $^{192}$Ir-UFP is translocated along different pathways. In fact, ultrafine particles may use different transcytotic pathways such as clathrin-coated pits, pinocytosis and non-coated pits, called caveolae (Gumbleton, 2001). Caveolae are the most likely route of uptake for the $^{192}$Ir-UFP used in our study (18 nm) as derived from studies by Gumbleton (2001). The alveolar epithelial cells, comprising 95 % of the lung surface have a

cell thickness of 400 nm and about 600.000 to 900.000 caveolae that are 50-60 nm wide. Such a high number of possible transport units lead to assumption that NP smaller than 60 nm can be rapidly taken up and transported through the epithelial cell layer. Recently Kato et al, (2003) showed that lecithin-coated polystyrene latex beads (240 nm) got incorporated into the Type I and II alveolar epithelial cells as well as in the capillary lumen. It was suggested that these latex beads move from the alveolar epithelial cells to the capillary lumen via transcytosis. Kapp et al (2004) found $Ti0_2$ (29 nm) particles as intracellular clusters forming needle shape particles or rounded shape particles. This may also explain the relatively low and variable translocation as observed in other, previous *in vivo* studies (Kreyling et al, 2002 ; Nemmar et al, 2002 ; Nemmar et al, 2002 Nemmar et al, 2002 ; Oberdorster et al, 2002). We therefore used histamine to modify or facilitate trans-endothelial passage and indeed found that this enhanced translocation of $^{192}$Ir-UFP to the perfusate. However, we cannot discriminate between transcytosis and para-cellular transport in endothelium. We assume that the long lag-time (60 min) detected which is needed before passage is caused by the fact that an interstitial load has to be built by epithelial passage of $^{192}$Ir-UFP in the lung. The methods used in our study and the study by Nemmar et al, (2005) do not allow to evaluate whether translocation has occurred through primary particles or by aggregates. The inhalation in our study assured single UFP deposition in the alveolar region and virtually no agglomeration on the epithelium because of the alveolar surface and the number of deposited particles. However, upon vascular injection aggregates are formed, unless surface modifications are used that impede this process. It may therefore be that translocation observed in Nemmar's study occurs as aggregates by above mechanisms, or facilitation by phagocytic cells but not in our study. With this respect the studies by Heckel et al (2004) have demonstrated by TEM that 4 nm gold-particles really pass membranes and reach the lumen as single particles. The difference between 4 and 20 nm particles may however be huge since 4 nm AU-particles are not recognized by the reticulo-endothelial system.

Our findings may be criticized due to a number of factors that are associated to our experimental design and performance. First, it must be taken into account that isolated and perfused lungs are not under physiological conditions since, for example, lymph flow is altered, bronchial perfusion is suppressed, autonomic innervation is disconnected and no blood cells (including inflammatory cells) are present in the perfusate. However the artificial negative pressure perfusion of isolated lung resembles respiratory conditions and the time span of experiments was limited up to 2 hours maximum to avoid excessive decrease of function. Knowing this, the lack of concomitant physiological measurement in the negative pressure ventilation is a major shortcoming in our data. The obvious reason for this is that given the use of radioactive $^{192}$Ir-UFP inclusion of measurement devices was allowed for

radiation protection safety reasons, since they could lead to an open system and radioactive particle emissions. We have tried to compensate for this lack of know-how by measurement of biochemical indices of damage in perfusate and performing histology in lung after the experiment. No evidence for extensive lung damage in control conditions or after in-situ treatment to the essential barriers of the lung was noted. The release of ALP and ACE as biomarkers of epithelial and endothelial integrity are not elevated during the perfusion and do not correspond to the small translocation of $^{192}$Ir after a lag-time of about 60 min -UFP. Although microscopical analysis in lung sections showed desquamation of the epithelial layer in the lungs treated in situ with hydrogen peroxide, ALP levels in perfusate were not different from that in the control group. The experimental conditions also did not affect the integrity of the endothelial layer since perfusate levels of ACE did not change during perfusion. However, in most lungs oedema was noted in microscopy as interstitial dilation (Table 1) at the end of the experiment. The formation of oedema is due to the imbalance between fluid transvascular filtration and clearance. Also the excess fluid causes an overhydration of the interstitium associated with accumulation of oedema fluid in the loose connective tissue [51]. It could be argued that oedema might affect the translocation of $^{192}$Ir-UFP from the lumen via the interstitium across the endothelium. In fact, a recent study using iv injection of colloidal gold particles (4 nm) in rabbits, showed that a small but significant percentage (7 %) of the UFP was taken up in endothelial and epithelial cells of the lung (Heckel et al, 2004). After LPS infusion, causing mild pulmonary oedema, transendothelial transport was boosted five-fold, while a significant amount of gold particles accumulated in the interstitium (14 %) and even reached the alveoli (11 %). Although this suggests a potential interference for oedema in our study, one should realize that in the above study (Heckel et al, 2004) oedema was present at the very beginning and translocation is followed in a different direction. Nevertheless an effect of oedema on particle translocation is considered unlikely in our experimental setup since no $^{192}$Ir-UFP translocation was observed in the control groups and after a histamine bolus for up to 2 hours after onset of inhalation.

The minute translocation of $^{192}$Ir-UFP in the isolated lung perfusion system conforms to our previous in vivo findings (Kreyling et al, 2002) using the same particles by inhalation at similar dose in rats. Other studies however reported very different amounts and kinetics of translocation. Nemmar et al, (2001) studied particle translocation after intratracheal instillation of uF particles in hamsters *in vivo* and observed a rapid (3 % within 5 min) translocation of 80 nm albumin particles coated with $^{99m}$Tc but no translocation was observed after the 15 minutes. On the other hand, the same group could not find latex (24-190 nm) particle translocation isolated perfused rabbit lungs at positive pressure (Nemmar et al, 2005). Surface chemistry did not affect this process. In a similar approach using latex

fluorescent beads and positive pressure ventilation, we also did not find particle translocation (data not shown). In contrast Brooking et al, (2001) showed a continuous increase in translocation with time up to 180 minutes during nasal inhalation of latex particles between 50 and 250 nm by rats. The latter study highlighted the importance of particle size as the smallest ultrafine particles showed higher uptake rates than the larger particles. In addition they demonstrated that particle surface chemistry was an important characteristic (Brooking et al, 2001). Oberdorster et al, (2002) reported quite extensive (> 20 %) translocation of uF carbon particles (18 nm) after short-term inhalation. Whatever the mechanism or particle properties involved in passing the lung barriers, the question remains what particle translocation means in terms of systemic effects. The mainstream hypothesis is that lung inflammation causes and facilitates the release of mediators that adversely affect cardiovascular parameters (Seaton et al, 1995). Alternatively, translocation of particles to the brain (Oberdorster et al, 2004) or the systemic circulation may also explain effects of PM exposure on heart and vascular tissue. The blood that leaves the lung first enters the heart before it is pumped to the other organs. In our previous work we showed that suspensions or filtrates of $PM_{10}$ could have direct effects on vessels (Bagate et al, 2004). However, the effects were rather due to the soluble components (i.e. transition metals) than to particles themselves (Bagate et al, 2004) and are in contrast to *in vivo* findings on endothelial function with PM (Brook et al, 2002; Nurkiewicz et al, 2004).

Although these data do not allow quantitative conclusions on the exact mechanism and the importance of systemic translocation of UFP as a mechanism in adverse effects of PM, we do confirm that ultrafine particles can translocate from the lung into the circulation using the isolated perfused rat lung upon pharmacological mediation. Permeability of the lung barrier to ultrafine particles seems to be controlled both at the epithelial and endothelial level and conditions that affect this barrier function such as inflammation may affect translocation of UFP. The conditions under which this does occur mimic conditions that are met in diseased, susceptible subjects including asthmatics and COPD-patients.

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## *Table 1*

Morphological evaluation of the rat lungs after 2 hours perfusion and inhalation exposure to Ir-UFP, using lung sections and HE- or toluidine blue staining The regions investigated included the bronchial segment, the blood vessels and the alveolar region.

| **Treatment** | **Bronchial region** | **Blood vessels** | **Alveolar region** |
|---|---|---|---|
| **Control** | Sub epithelial round cell filtration; interstitial dilation | Interstitial dilation | Small alveolar dilation in periphery |
| **Hydrogen peroxide** | Desquamation of epithelial layer, partially destruction of sub epithelial structure | Moderate to severe dilation | |
| **Histamine Perfusate** | Sub epithelial round cell filtration; interstitial dilation | Moderate to severe interstitial dilation | Alveolar dilation in periphery |
| **Histamine (Bolus)** | Sub epithelial round cell filtration; interstitial dilation | Moderate to severe interstitial dilation | Focal alveolar dilation, oedematous fluid in alveolar lumen |

## FIGURE LEGENDS

**Figure 1.** Diagram of the experimental perfusion system used for this study. The ultrafine iridium particles (Ir-UFP) radiolabelled with $^{192}$Ir were produced in the spark generator. At the exit of spark generator the aerosol was quasi-neutralized by a radioactive $^{85}$Kr source. The aerosol was diluted with nitrogen and with oxygen and adjusted to obtain 20% oxygen and was air conditioned at 50-60 % relative humidity. The particle size distribution and number concentration were monitored by a differential mobility particle sizer (DMPS) and a condensation particle counter (CPC). $^{192}$Ir-UFP radioactivity of the aerosol was determined by continuous aerosol sampling of a measured volume and integral radioactivity counting. The lungs were perfused at a perfusion rate of 5 ml/min and a stroke volume of 10 ml. Respiration rate set at 65 breaths per minute. Negative ventilation pressure in chamber was regulated with animal ventilator. Lungs were manually expanded (sighed) every 4 minutes by applying a negative pressure of –2.5 kPa to the chamber.

**Figure 2.** Experimental protocol for isolated perfused rat lungs. All perfusions were done under negative pressure ventilation. Following a 15 minute period of equilibration, during which the lungs were already ventilated and perfused, the experiment started by the inhalation of freshly produced $^{192}$Ir-UFP from the aerosol line. Intratracheal instillation of $^{99m}$Tc-DTPA or other instillations were performed at this starting time point. The perfusate was collected continuously and sampled at 15-min time intervals. The following treatments were investigated, *group 1*: control group, only $^{192}$Ir-UFP inhalation for 120 min; *group 2*: instillation of 50-100 µL $^{99m}$Tc-DTPA, 500 µl $H_2O_2$ bolus (0.5 mM), $^{192}$Ir-UFP aerosol inhalation for 120 min; *group 3:* instillation of 50-100 µL $^{99m}$Tc-DTPA, histamine continuously pefrused during the next 2 hours at concentration 10 µM, $^{192}$Ir-UFP aerosol inhalation for 120 min; *group 4*: instillation of 50-100 µL $^{99m}$Tc-DTPA and 500µl histamine bolus instillation at a concentration of 10 mM, $^{192}$Ir-UFP aerosol inhalation for 120 min; *group 5*: instillation of 50-100 µL $^{99m}$Tc-DTPA. For each group 3-4 animals were used.

**Figure 3**. Average particle size distribution in a typical perfusion experiment over entire exposure time (120 min), revealing an average size distribution of count median diameter of16.9 nm, GSD of1.6, aerosol concentration 4.45 $10^6$ $cm^{-3}$, SD 0.13 $10^6$ $cm^{-3}$ The example shown is from inhalation during histamine perfusion, as shown in Fig 3 (lower panel).

**Figure 4.** Translocation of $^{192}$Ir-UFP particles (upper panel) and $^{99m}$Tc-DTPA (lower panel) into perfusate of isolated perfused rat lung as a fraction of the deposited dose for inhaled particles and as fraction of the instilled dose for DTPA. Both control lungs (•) were used as well as lungs treated in-situ with $H_2O_2$ bolus 0.5 mM (O), histamine (1 μM) in perfusate (□) or 0,5 mM instilled into the lungs (■). A stabilisation period of 15 minutes is done before treatment and collection of samples are taken place every 15 minutes for translocation and lung markers detection. Values in the upper panel depict the mean and SD's of 3 or 4 experiments; values in the lower panel depict only the mean of 3 or 4 experiments indicating the trend**.**

**Figure 5.** Translocation of Iridium particles in individual perfusions after treatment with (A) $H_2O_2$ (bolus) and (B) histamine (10 μM) in the perfusate. Translocation represented as relative radioactivity of $^{192}$Ir in the perfusate. Data shown for individual heart-lung-blocs.

**Figure 6**. Release of Alkaline phosphatase (A) and Angiotensin converting enzyme (B) measured in lung perfusate during and after particle inhalation and different pre-treatments. Both control lungs (•) were used as well as lungs treated with $H_2O_2$ (O), histamine (10 μM) in perfusate (□) or injected into the lungs (■). Values depict the mean of 3 or 4 experiments.

**Figure 7.** Examples of histopathological lesions encountered in lungs after 2 hour perfusion and inhalation of non-radioactive $^{192}$Ir-UFP in control lungs (A), lungs pretreated with a bolus of H2O2 in the lumen (B, D), showing septum and bronchus reduction, and (C) lung perfused with histamine, showing oedema and infiltration.

**Figure 1.**

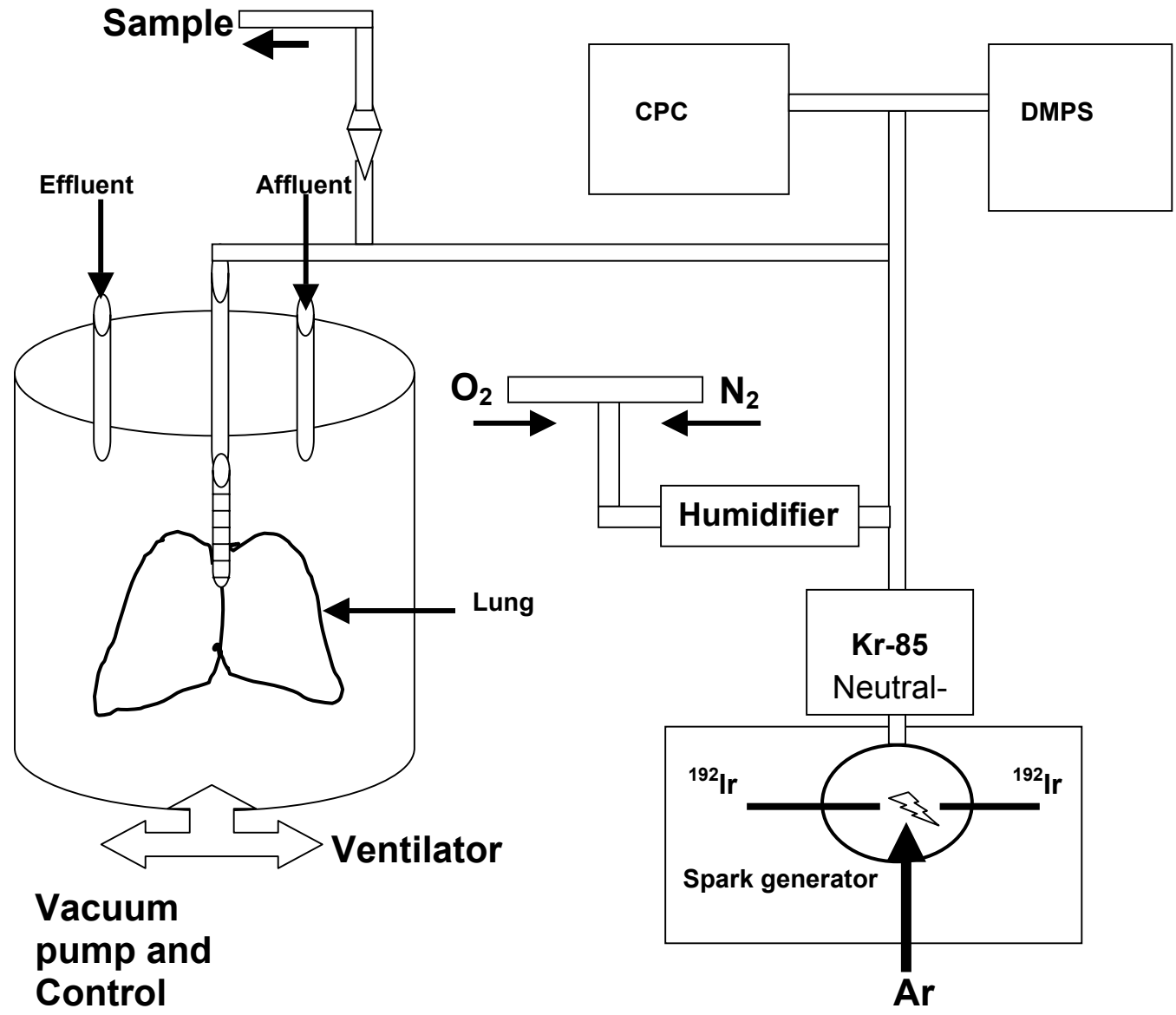
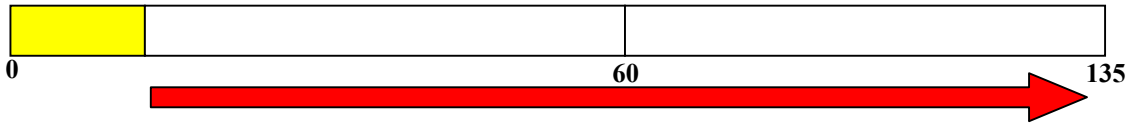
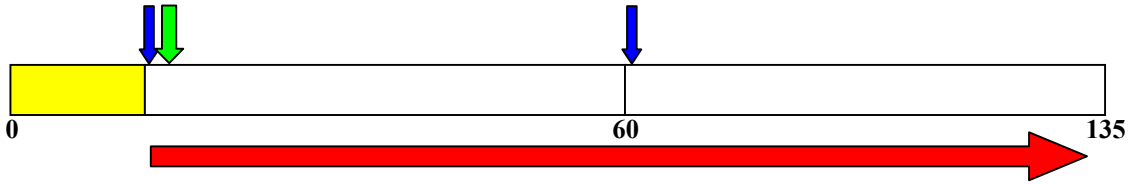

Sample
CPC
DMPS
Effluent
Affluent
$O_2$
$N_2$
Humidifier
Lung
Kr-85
Neutral-
$^{192}Ir$
$^{192}Ir$
Spark generator
Ventilator
Vacuum
pump and
Control
Ar

**Figure 2**

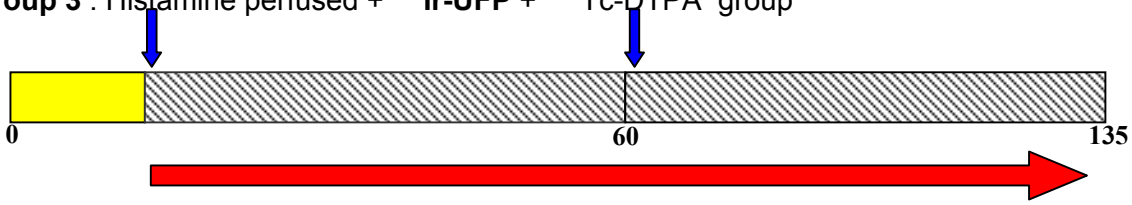
**Group1** : **Control group.** $^{192}$**Ir-UFP**



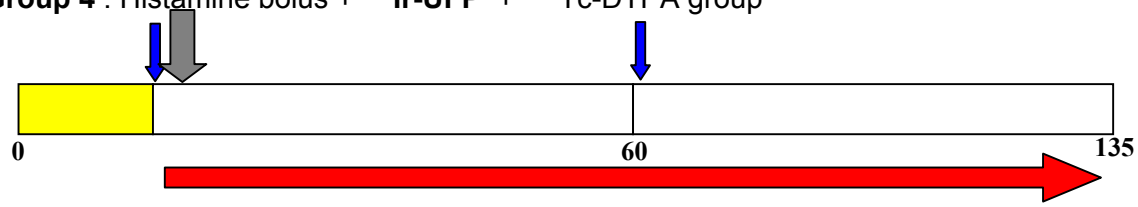

**Group 2** : **$H_2O_2$** bolus + $^{192}$**Ir-UFP** + $^{99m}$Tc-DTPA group.



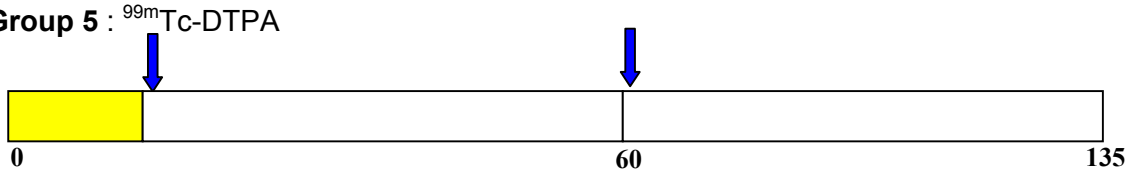

**Group 3** : Histamine perfused + $^{192}$**Ir-UFP** + $^{99m}$Tc-DTPA group




**Group 4** : Histamine bolus + $^{192}$**Ir-UFP** + $^{99m}$Tc-DTPA group




**Group 5** : $^{99m}$Tc-DTPA





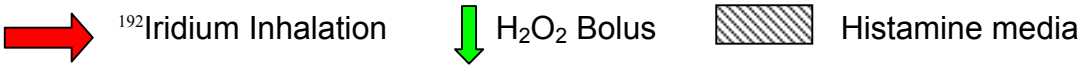


**Figure 3**

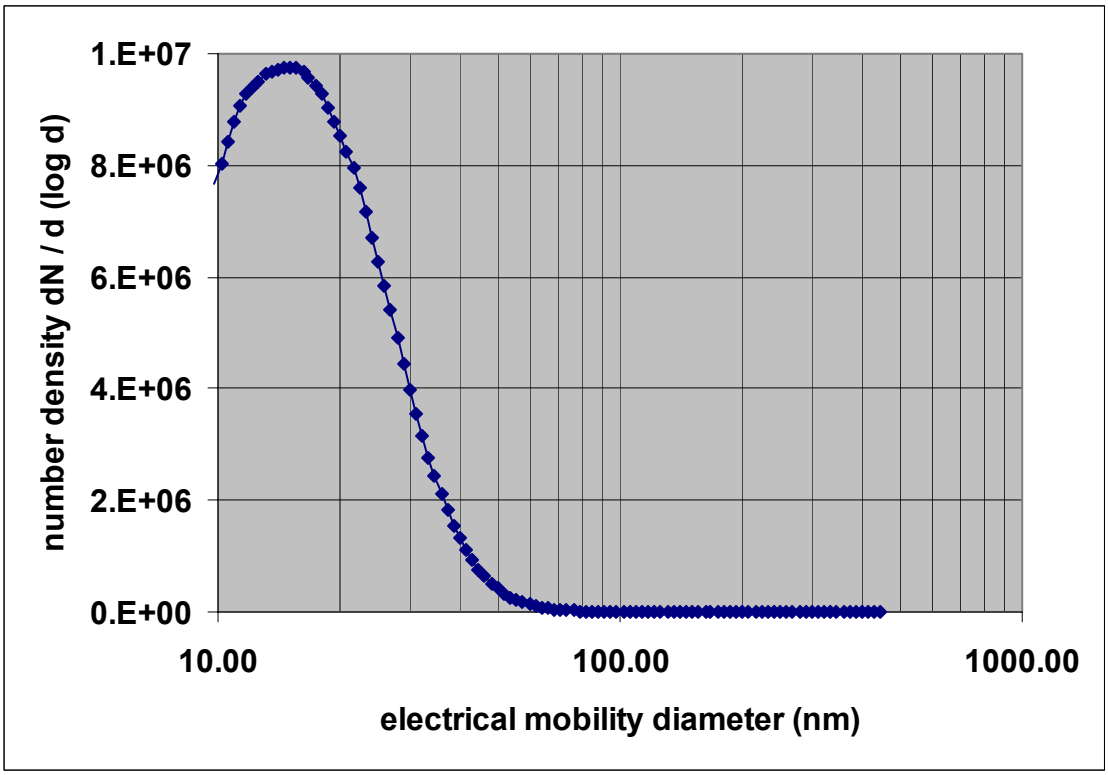
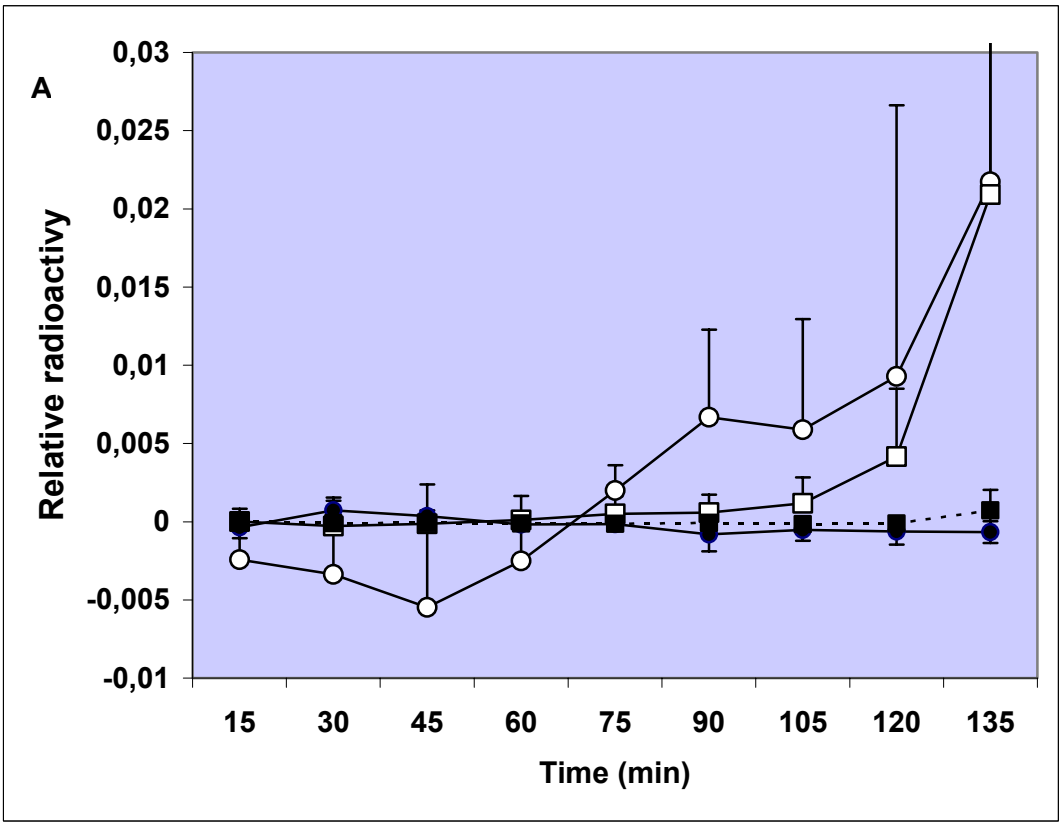

1.E+07
8.E+06
6.E+06
4.E+06
2.E+06
0.E+00
number density dN / d (log d)
10.00
100.00
1000.00
electrical mobility diameter (nm)

**Figure 4**


A
Relative radioactivy
0,03
0,025
0,02
0,015
0,01
0,005
0
-0,005
-0,01
15
30
45
60
75
90
105
120
135
Time (min)

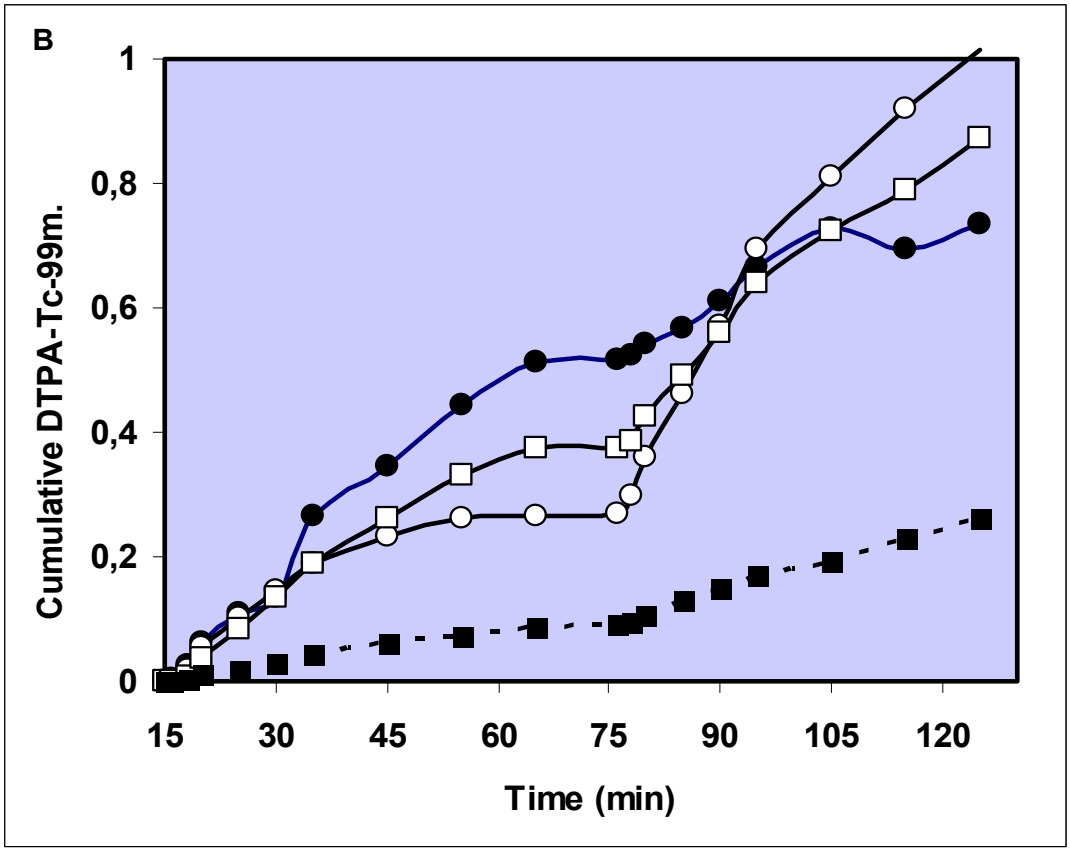

B
Cumulative DTPA-Tc-99m.
1
0,8
0,6
0,4
0,2
0
15
30
45
60
75
90
105
120
Time (min)

**Figure 5A**

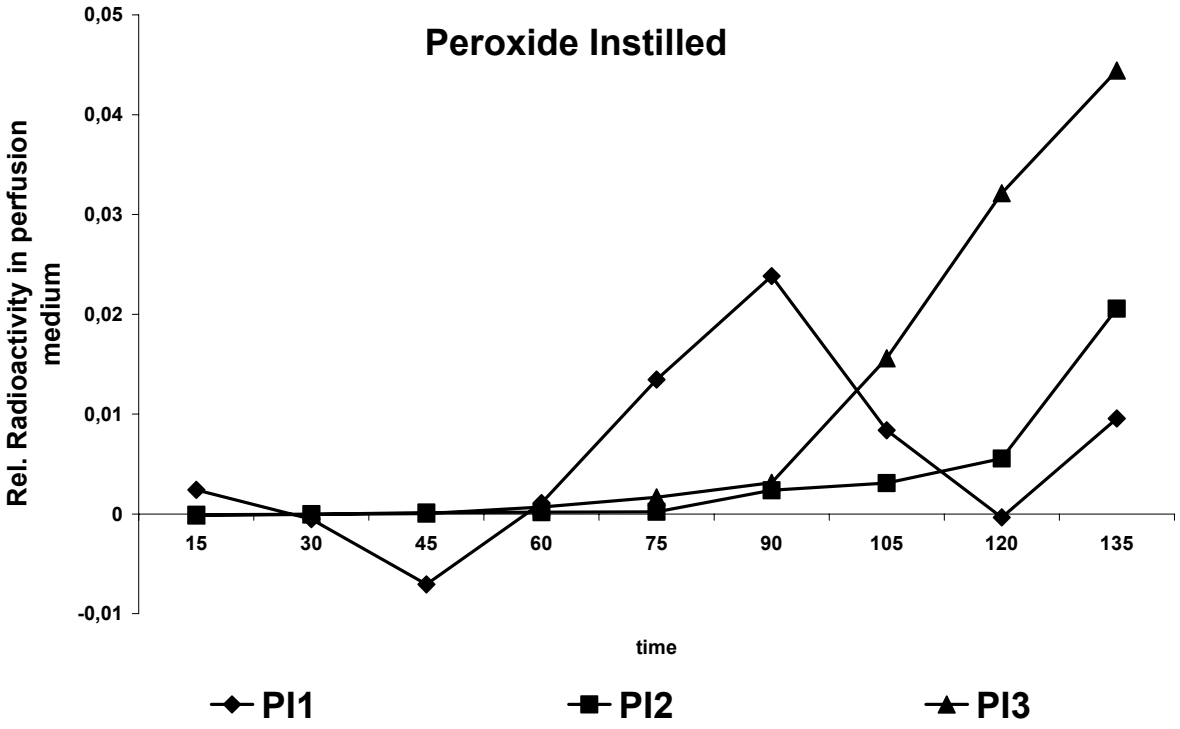



**Figure 5B**

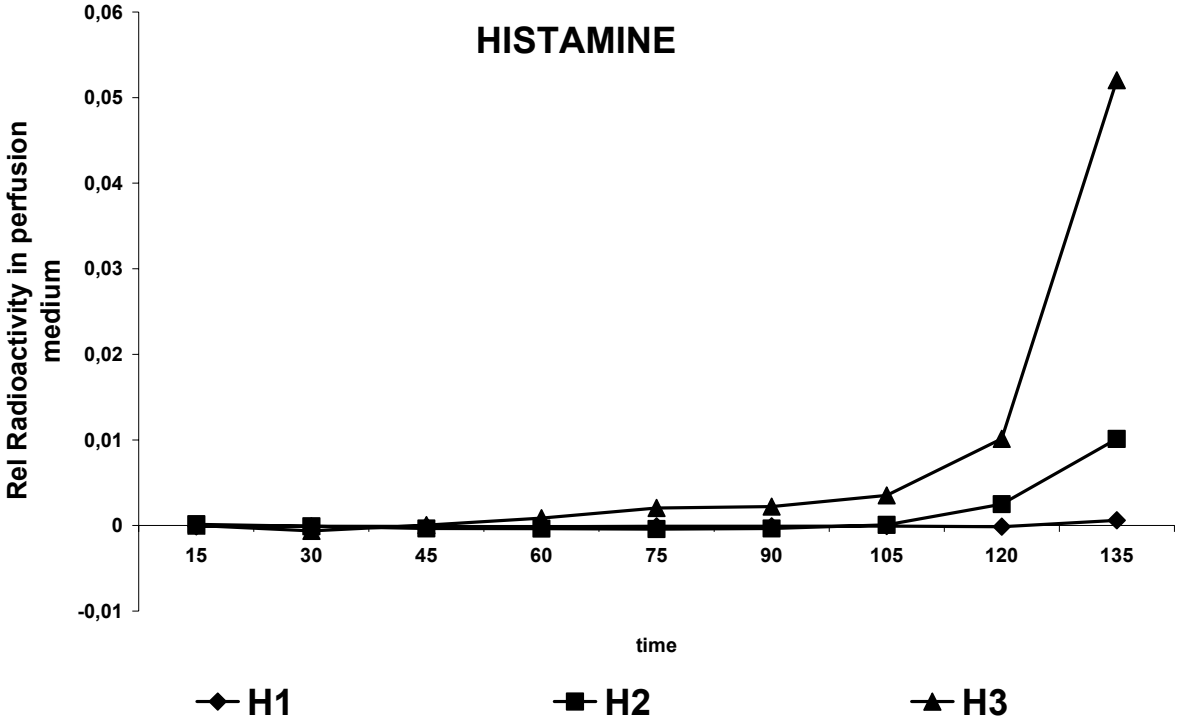


**Figure 6A**

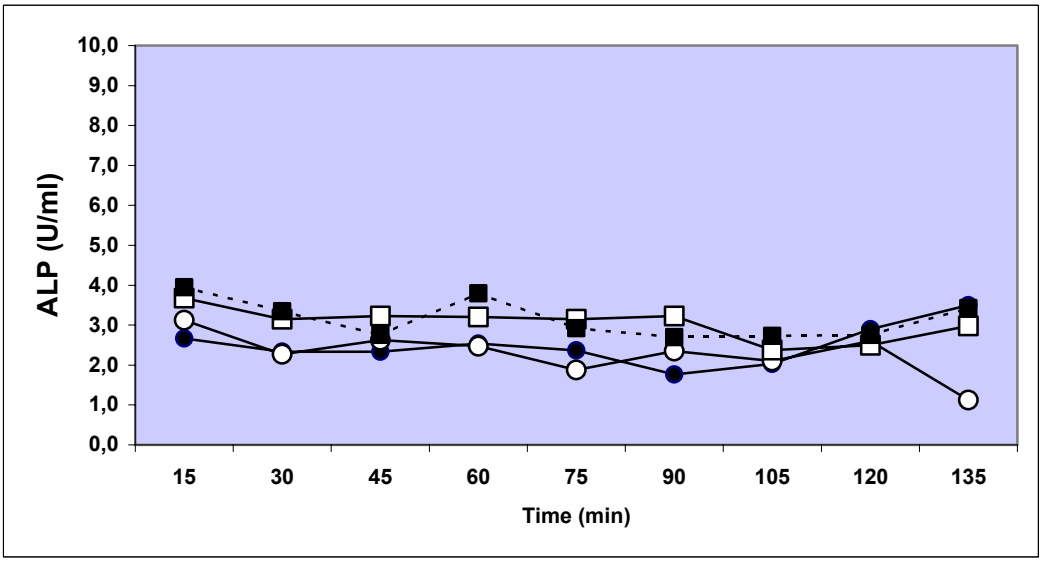



**Figure 6B**

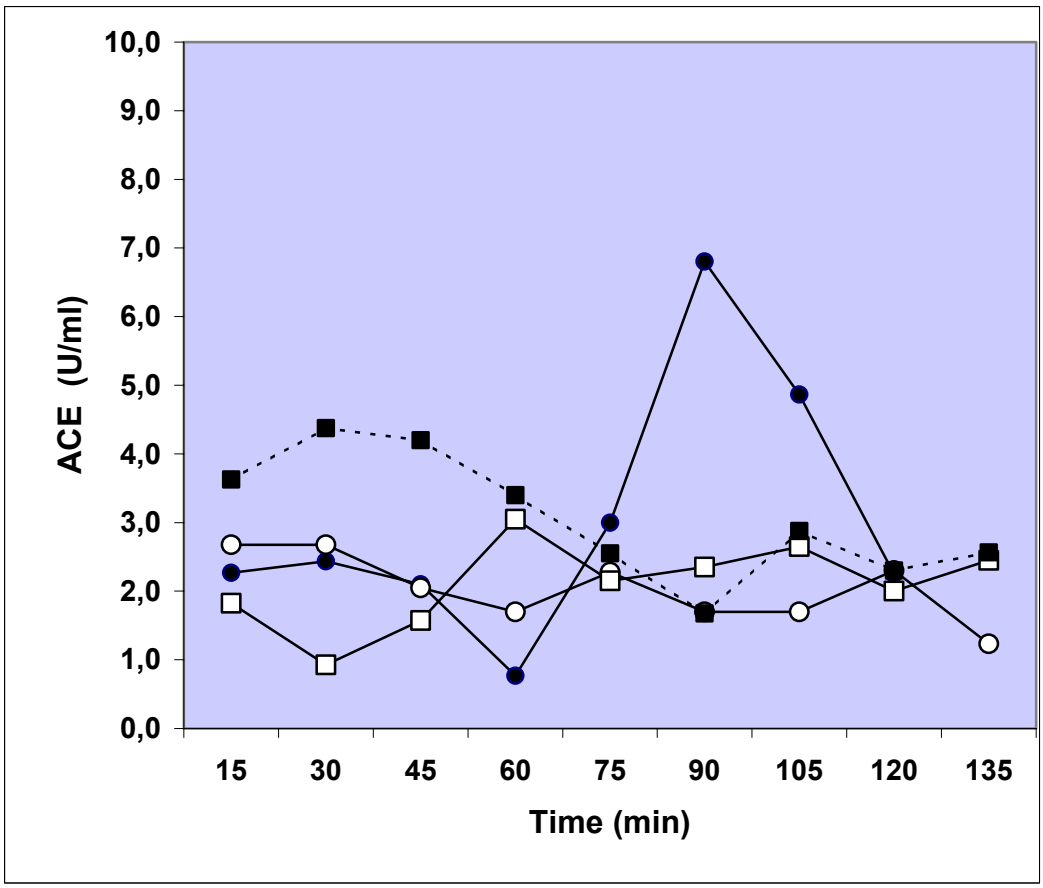
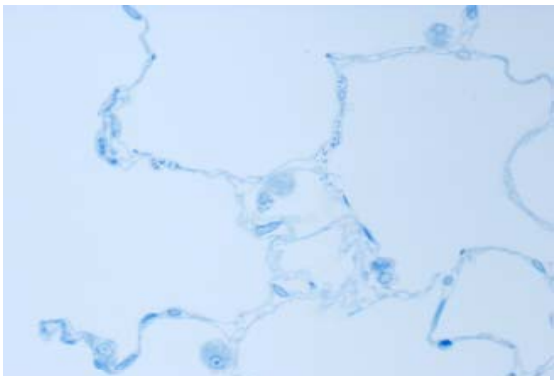
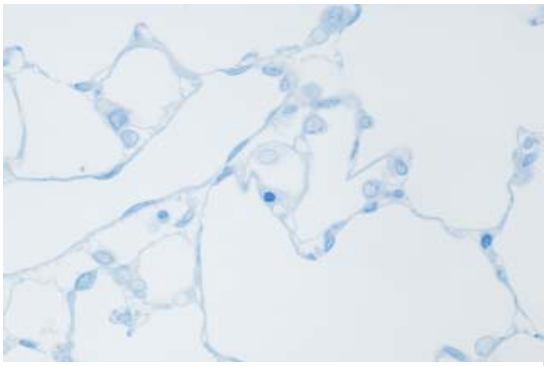


**Figure 7**

**A**



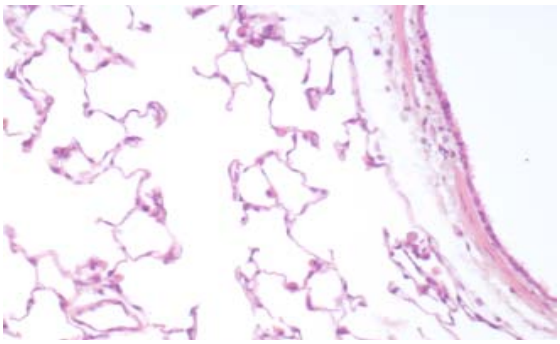
LP- Ir- No pretreatment



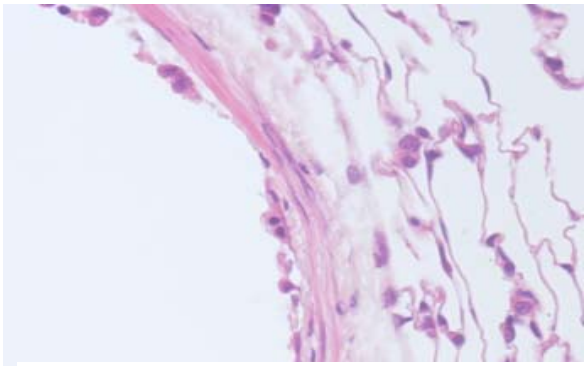
Ir- H2O2-pretreatment
septum reduction

**B**

**C**



LP- Ir- Histamine (perfusate)
oedema + infiltration



Ir- H2O2-pretreatment
bronchus reduction

**D**

## CHAPTER 3

# Neutrophil influx as model of inflammation in the isolated perfused rat lung and its effects on nanoparticle translocation

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## Abstract

**Background**. Pulmonary Inflammation increases after particle exposure and this process might affect pulmonary permeability. The purpose of the study was to evaluate the role of epithelial and endothelial barrier function in the translocation of ultrafine particles across the lung in to the systemic circulation. Our main objective was to produce a physiological model of PMN influx in the isolated lung perfuse lung model and evaluate the effects of PMN on the translocation of $^{192}$Ir-UFP particles.

**Methods.** The isolated perfused rat lung (IPRL) was used under positive pressure ventilation, and radioactive iridium particles (18 nm, CMD, $^{192}$Ir-UFP) were instilled intratracheal after 15 minutes of stabilisation of lung. Particle translocation was evaluated after instillation of $^{192}$Ir-UFP in lungs perfused with histamine, PMN enriched media with and without activation (FMLP) and combinations of both treatment. PMN were labelled with the fluorescent dye CFSE to allow visualisation of PMN influx in the lung interstitium Enzymatic analysis of mediator release was performed such as alkaline phosphatase (AKP) and Angiotensin converting enzyme (ACE) to assess epithelial and endothelial integrity.

**Results**. Particle translocation was not observed in any treatment, which contrasts to our earlier findings where lungs were exposed by inhalation to the $^{192}$Ir-UFP. Although lungs perfused with activated PMN didn't reveal particle translocation, the IPRL model can be used for further neutrophil studies in isolated perfused lung models. **Conclusion:** Particle translocation in a positive pressure IPRL with instillation of particles seems to be not a suitable model to explain the translocation effects of particles. The negative pressure IPRL model is thus the ideal model to investigate particle translocation.

**Keywords**: endothelial, epithelial, translocation, ultrafine particles, histamine, hydrogen peroxide

# 1. INTRODUCTION

Apart from a relationship between short-term increases in $PM_{10}$ in time-series studies and deaths from cardiovascular/ cardio-respiratory causes ( Hoek *et al,* 2001; Peters et al, 2001; Zanobetti et al, 2000), also early biological responses of the cardiovascular system have been reported. Both blood pressure, heart rate variability and heart rate were found to be associated to model and real life PM ( Dockery et al, 1999; Brook et al, 2002). These data suggest that PM has a short-term effect which triggers a reflexive change in cardiac function and myocardial stimulation via the autonomic nervous system. However, a plausible mechanism to explain for these effects is still lacking. The possibility of transfer of particles and/or vasoactive mediators (such as endothelin) via the blood from the lungs to the heart seems likely, but the data in this regard is limited and confusing (Nemmar et al, 2001, 2002; Kreyling et al, 2002). Most of the inhaled dose of particles remains in lung interstitium (Ferin et al, 1992), thus raising the question that translocation of particles is slow and by the endothelial barrier. We have found increased particle translocation after perfusing an isolated lung with histamine causing the endothelial layer to become more permeable (Meiring et al, 2005). Nemmar et al, (2004) found that particles cause the release of vasoactive mediators such as histamine which was shown to be increased in plasma of guinea pigs after instillation of diesel exhaust particles. Histamine is well known for inducing vascular permeability through its action on endothelial H1 receptor. Inhaled polymorphonuclear leukocytes (PMN) induce pulmonary oxidative stress and inflammation (Donaldson et al, 2003). The inflammation process is accompanied with the migration of PMN through the epithelial layer and can be detected in bronchoalveolar lavage (BAL) fluid. There is substantial evidence that during this inflammatory process the endothelial layer in the lung is the first target for the leukocytes. Collard et al, (2002) stated that endothelial barrier function is altered by PMN adhesion during an inflammatory state. Huang et al, (1997) showed that lung vascular permeability is affected by activated PMN's. The vascular barrier function might also be affected by the release of soluble mediators from the activated polymorphonuclear leukocytes (PMN) (Cotran et al, 1998). The influx of inflammatory cells causes the release of mediators such as histamine, as well as $H_2O_2$. These together with mediators such as adenosine (Lennon et al, 1998) and glutamate (Collard 2002) may influence the endothelial barrier. In addition, neutrophil elastase has the ability to disrupt elastic network of tissues, as well as the extracellular matrix (Dona et al, 2003). Finally reactive oxygen species (ROS) also have a direct effect of cellular membranes by lipid peroxidation and nitrosylation of proteins (Choudhary and Dudley, 2002). As such neutrophil-led inflammation has been

shown to affect endothelial barrier function in various chronical lung diseases ranging from bronchitis, asthma, emphysema and fibrosis to tumor progression.

The general design of our studies is driven by the concept that if particles or particle components (metals) are able to cross the epithelial barrier in the lung, and reach the heart and aorta they should be able to induce direct pharmacological effects. In this context we recently showed impairment of vascular function after instillation of ambient particulate matter in rats (Bagate et al, 2004) We also showed in isolated lung perfusion that $H_2O_2$ instilled into the alveolar lumen was able to induce the translocation of ultrafine Iridium-particles ($^{192}$Ir-UFP). Since $H_2O_2$ is one of the products released by neutrophils and PMN influx may affect epithelial and endothelial permeability, we set-out to investigate the role of PMN in facilitating the translocation of ultra fine particles in the lung. To address this question we made use of the isolated perfused rat lung (IPRL), using a positive pressure system and administered $^{192}$Ir-UFP particles (18 nm) by instillation into the trachea. This IPL allows us to exclude systemic anti-oxidant back-up from the perfusing blood, to imitate an inflammatory state using PMN migration into the tissue and alveolar space as well sample perfusate that can be investigated for specific biological mediators release during particle treatment.

## Materials and methods

### Chemicals

Hank's balanced salt solution (HBSS), dimethyl sulphoxide (DMSO), lucigenin, phosphate buffered saline (PBS), phorbol-12-myrisate-13-acetate (PMA), N-formyl-Met-Leu-Phenylalanine (fMLP), Fluorescein 5(6) isothiocyanate were also purchased from Sigma. Lymphoprep was purchased from Nycomed, 5-(and-6)-carboxyfluorescein diacetatesuccinimydyl ester (CFSE) purchased from Boitrend Chemikalien GmbH (Köln) Germany

### Animals and animal husbandry

Male Wistar-Kyoto rats (WKY/Kyo@Rj rats, Janvier, France) (250-350 g) were used in this study. They were housed in pais in humidity (55% relative humidity) and temperature (22$^0$C) controlled room. They were maintained on a 12-h day/night cycle. Rats were allowed to acclimate to the facility for a minimum of 10 days prior to use. For establishment of this project, rats were more than 17 weeks of age were used. The studies were conducted under federal guidelines for the use of laboratory animals and were approved by the Regierung von Oberbayern and by the GSF Institutional Animal Care and Use Committee.
Surgical procedure was done according the method of Uhlig and Wollin (1994). The same procedure was followed as in **Chapter 2**.

**Preparation of PMN (for circulating lung perfusion)**

Human poly morphonuclear leokocytes (PMN) were freshly isolated from venous blood (S-Monovette, 1,6 mg EDTA/ml blood, Sarstedt) from healthy volunteers. The preparation of the PMN included sedimentation, Lymphoprep (Nycomed) density centrifugation at 2500 rpm and osmotic lysis of leukocytes in a Lysis buffer. The lysis buffer consist of 155 µM $NH_4Cl$, 10 mM $KHCO_3$ and 1 µM EDTA. All solutions were kept at 4 $^0$C to prevent premature PMN activation during the isolation procedure. PMN were washed twice and suspended in Hank'' balanced salt solution. Cell viability was assessed by trypan blue exclusion and the preparation of PMN was about pure and 96% viable. PMN were labelled with the fluorescent dye CFSE (5 and-6 carboxyfluorescein diacetatsuccinimydyl ester) by incubation in CFSE (1 µM) containing HBSS for 15 minutes. Washing and centrifugation (1200 rpm) were done to remove adherent dye and non-viable cells.

**Lucigenin-enhanced chemiluminescence**

Naïve or CFSE loaded human neutrophils were exposed to PMA or fMLP after storage at different temperatures and superoxide release was assessed using lucigenin-enhanced chemiluminescence. Human neutrophils were isolated as described previously and suspended in HBSS at 2 x $10^6$ cells/ml. 100 µl of the Neutrophil cells suspension 100 µl lucigenin (5x $10^{-4}$M) was added. PMA (100ng/ml) was used as a positive control. Lucigenin-enhanced chemiluminescence was then recorded during 40 min using a luminometer (Multi-Bioluminat, Berthold, Germany) at 37 $^0$C and expressed as area under the curve. All stimulatory agents and appropriate controls were tested in parallel and the experiments were repeated 4 times.

**Chemotaxis assay in PMN**

Venous blood (S-Monovette, 1,6 mg EDTA/ml blood, Sarstedt) was collected and neutrophils were isolated as described above. Chemotaxis and random migration were evaluated with a 96 well microchamber using a Neuroprobe AB96 96-well micro-Boyden chamber as described by Drost et al. (2002). We used a polycarbonate filters with 3 µm diameter pores. In the bottom wells we placed HBSS, fMLP ($10^{-6}$, $20^{-6}$ and $30^{-6}$ M) and IL-8 ($10^{-9}$ , $10^{-10}$ and $10^{-11}$ g/ml ). Neutrophils pre-treated with PBS or loaded with CFSE-dye were added to the

top wells of the chamber. Each treatment was tested in triplicate. The chamber was incubated for 30 minutes at 37 $^{o}$C, 5% $CO_2$. After the incubation the chamber was undone and the polycarbonate filter was removed, air dried and the neutrophils were fixed and stained with May Grunwald and Giemsa respectively. The neutrophils which were adherent to the top of the filter and had migrated into the filter (adhesion and migration) were measured using a microplate reader (Labsystems –MultiSkan Ascent) at an absorbance of 550 nm. The data were expressed as average absorbance values from which the background absorbance values for the filter alone had been subtracted.

**Lung perfusion**

**Ventilation/perfusion apparatus**

The same IPL-4401 Isolated lung ventilation perfusion system (FMI GmbH Oberbach) as describe in **Chapter 2** was used for this study.

**Aerosol production and_characterization**: Aerosols of ultrafine iridium particles radio-labeled with $^{192}$Ir were generated with a spark generator (GFG 1000, Palas). The radio isotope $^{192}$Ir is a beta and gamma emitter with a half-life of 74 d and gamma energies of 296, 308, 316, 468, 588 keV (26, 29, 73, 47, 5% efficiency, respectively); there are also some higher and some lower energies of low efficiency; gamma spectroscopy was performed using the photo peaks of 296, 308, 316 keV. Sparks of constant energy were ignited between neutron-activated pure iridium electrodes at a frequency of either 3 or 15 Hz in an argon stream of 3.5 L/min. At the exit of the spark generator the aerosol was quasi-neutralized by a radioactive $^{85}$Kr source (40 MBq). The aerosol was diluted with nitrogen and – for rat exposures – with oxygen adjusted to obtain 20% oxygen and was air conditioned at 50-60 % relative humidity and 37°C for rat exposure. Size distribution and number concentration was monitored continuously by a differential mobility particle sizer (DMPS; classifier mod.3070 and CPC mod. 7610, TSI) and a condensation particle counter (CPC3022A, TSI), respectively. Lower particle size detection limit of the former was 10 nm. $^{192}$Ir radioactivity of the aerosol was determined by continuous aerosol sampling at a flow rate of 0.2 L/min, volume measurement and gamma counting in a well-type scintillation detector. Aerosol mass concentration was derived from the given specific activity of the electrodes.

**Perfusion media**

The perfusion medium was selected based on its extensive use in isolated organ perfusion and consisted of a filtered modified Krebs-Ringer –bicarbonate buffer. Research have shown that salt buffers must be substituted with plasma expanders to prevent edema formation in the lung (Uhlig and Wolling 1994). Krebs-Ringer composition was as follows (mM): NaCl 118 ; KCl 4.7 ; $CaCl_2$ 2.5 ; $MgSO_4$ 1.2 ; $KH_2PO_4$ 1.2 ; $NaHCO_3$ 13 ; 0.1 % glucose , 0.3 % HEPES and 2 % albumin (fraction V standard grade from Serva , Heidelberg, Germany) pH was adjusted at 7.4. The buffer was pre-warmed and gassed with 95% $O_2$ and 5% $CO_2$ at a rate low enough to prevent excessive frothing of the medium. The medium flowing through the system passed through a bubble trap prior to reaching the lungs and the buffer pH was continuously monitored throughout the experiment. The final concentration of PMN was made up of a suspension of $1x10^6$ cells /ml in the 50 ml recirculating volume buffer. The $1x10^6$ PMN/ml perfusate was introduced after 15 minutes of calibration with normal Krebs-Henseleit buffer, and re-circulating throughout the experiment.

**Experimental design**

Following a 15 minute stabilisation period, the lungs were subjected to different experimental conditions. The experiments (Figure 1) consist of 5 groups. I) control group with distilled water instillation at time point 15 minutes, II) Histamine perfusate with radioactive Iridium particles instilled intratracheal III) Normal Intratracheal instillation of radioactive Iridium particles IV) PMN enriched media with radioactive Iridium particles instilled V) PMN enriched media along with fMLP (neutrophils activator ) and radioactive Iridium ($^{192}$Ir) particles instillation. VI) Histamine and PMN enriched perfusate with radioactive Iridium ( $^{192}$Ir) particles. Radioactive particles instilled at time-point 15 minutes except controls. Perfusate collection was done every 15 minutes for determination of mediator release. The starting PMN concentration in the perfusion medium was $1x\ 10^6$ cells/ml.

**Tissue preparation**

Immediately after the termination of the lung perfusion the radioactive particles treated lungs were air dried with room air at a pressure of 3,5 kPa and subsequent imaging for particle distribution. For histopathology only lungs treated with non-radioactive iridium particles were used. After the experiment, the trachea and pulmonary vein of the IPL were perfused with 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7,2, 340 mOsm) at 25 cm fixative pressure. Postfixation of the same non radioactive lungs were done with the same fixation solution for 2 hours at room temperature and 25 cm fixative pressure. The lung lobes were then cut into 3 mm cubes with a razor blade. The lobes were then further fixed in 2 % glutaraldehyde in

0.1 M phosphate buffer, pH 7.2 for 2 hours at 4 degrees. Afterwards the cubes were shake for an hour at room temperature. A washing step of 1 hour followed with 0.1 M phosphate buffer. The lung cube probes were then embedded. Sections were viewed with fluorescence microscopy. The wavelengths to detect the labelled PMN's were exitation 495 and emission 519.

**Perfusate analysis**

Perfusate samples were stored at –20 °C before assessment of mediators including Angiotensin converting enzyme (ACE), alkaline phosphatase (ALP), and Myeloperoxidase (MPO). ACE was measured according the method of Maguire and Price (1985) using ACE standards from Bühlmann Labaratories AG, Switzerland. (Reference KK-ACK) The ACE activity was measured by measuring photometrically the hydrolysis of Furylacrylylphenalalanylglycylglycine (FAPGG). Determinations were done with samples at 37 °C using a Beckman DU 640 spectrophotometer. Alkaline phosphatase (ALP) was assessed using a kit from Merck (Cat no. 104019990314) and samples were measured at 25 °C on a Beckman DU 640 spectrophotometer. Protein determination was done according the Bicinchoninic acid (BCA) protein assay (Smith et al, 1985). Myeloperoxidase (MPO) activity, a marker of neutrophil activation, was determined according the method Klebanoff et al (1984), and described previously (Knaapen et al, 2002). Briefly, 200 µl samples were added to 800 µl assay solution. The assay solution was made up freshly and consisted of 19,2 µl Guaiacol ; 10,76 ml $H_2O$ ; 1,2 ml of 0,1M sodium phosphate buffer and 40 µl of 0,1 µM $H_2O_2$. The production of tetra-guaiacol was spectrophotometrically determined at 470 nm for 5 min. ( $\varepsilon$ = 26,6 $mM^{-1}$ at 470 nm )

**Statistical analysis**

Data are expressed as mean ± S.E.M., unless stated otherwise. Statistical analysis analysis was performed using SPSS version 10 for Windows. Differences were considered to be statistically significant when $P < 0,05$.

# RESULTS

### Effect of loading dye on PMN -function

The effects of storage time and temperature on neutrophil function were investigated on two vital neutrophil functions, i.e. oxidant production and migration. First, the optimal CFSE concentration was determined from experiments incubating PMN suspensions from different subjects with increasing concentrations of CFSE (0- 1μM). Both baseline and PMA induced ROS production was slightly affected up to 1 μM (Figure 1), while fluorescence did not increase. Subsequent experiments were therefore carried out with PMN pre-loaded in 0,1 μM CFSE. The ROS production of isolated neutrophil suspensions were evaluated after pre-loading PMN with CFSE and storage at either 37 $^{0}$C or 4 $^{0}$C. The neutrophils stored under 37 $^{o}$C showed a higher baseline and PMA induced ROS production compared with the neutrophils stored at low temperature (Figure 2). On the other hand, PMN that were stored at 37 $^{0}$C showed a rapid decline of function over two hours of storage than those stored at 4 $^{0}$C. In fact baseline ROS production fell about 50 % over 2 hours, and PMA stimulated more than 70 % over 2 hours of storage at 37 $^{0}$C ( Figure 3). This experiment therefore allows estimating neutrophil function during the two hours of perfusion in isolated perfused lung at 37$^{0}$C. PMN stored at 4 $^{0}$C showed no significant decline in ROS production, and therefore PMN suspensions were kept on ice before injection into the IPL. Evaluating labeled PMN vs unlabelled PMN‘s in the migration behaviour. Untreated PMN showed migration with various activators but the expected dose-response with fMLP and IL-8 showed an inverse response, indicative of a maximal effect at low concentrations. The unlabelled PMN showed a dose response with IL-8 and fMLP (Data not shown).

### PMN function in isolated perfused lung system

In order to check the activation status of PMN after injection into the lung perfusion system we measured the protein content and activity of myeloperoxidase in perfusate. Control experiments (no particle inhalation) were done and showed no detectable MPO activity from endogenous PMN in rat lungs. After injection of PMN into the perfusate to $10^{6}$ PMN per ml, a steady increase in MPO is observed (fig 4 A). From Figure 4A it can be seen that MPO activity increases after perfusion with PMN enriched medium. No MPO activity is visible in the control perfusion. Subsequent experiments were done to investigate if the PMN’s were already fully activated. Therefore samples of medium were lysed (Figure 4 B) before the end of the perfusion.

**PMN function and entrapment in IPL**

MPO activity was also used to evaluate the activation of the PMN during particle instillation in our isolated lung perfusion model. In figure 4, a MPO profile of the different experimental groups can be seen. The Iridium/PMN/fMLP group showed a higher MPO release compare with other group. For endothelial damage we measured angiotensin converting enzyme (ACE) from the perfusion samples. No significant differences were observed from the different experimental groups. The same was done with alkaline phosphatase (AKP) which is a marker for epithelial damage. Protein release was similar for all experimental groups.

**Translocation of ultrafine particles**

To investigate if $^{192}$Ir ultra fine particles can pass through the epithelial/endothelium barrier of the lung, translocation was evaluated using radioactive counts in perfusate sampled at different intervals. In none of the treatments (see Table 1) radioactivity was detected in the perfusate including lungs treated with PMN, fMLP and histamine. Several treatments were applied to investigate the role of epithelial and endothelial integrity as well as neutrophil influx on particle translocation. Figure 5 shows the radioactivity in lung and perfusate. A special set of experiments were designed to evaluate if the lung took up $^{192}$Ir-UFP particles from the circulation. The reason to do so was that in these experiments re-circulation of medium was applied, and we wanted to exclude that translocated particles can be trapped in the lung upon re-circulation. The absorption of the iridium particles onto the lung was not detectable (data not shown).

**Histology of lungs**

Histological sections were made of perfused lungs to locate the labeled PMN's in the lung. The PMN's were distributed evenly across the different lungs lobes. Figure 6 A, B, C shows the different sections of lungs perfused with CFSE labelled PMN's. However, due to auto fluorescence of the lung structures and distribution of the labelled PMN, no quantitative assesment could be done.

## DISCUSSION

Ambient particulate matter exposure has been implicated in epidemiological studies to be responsible for adverse health effects (Daniels et al, 2000; Pope et al, 2002). Various hypotheses exist but no clear biological mechanism or pathway is at hand to explain the health observed in epidemiological studies. Combustion derived nanoparticles have been forwarded as an important component in PM to cause these effects (Donaldson et al, 2005). CDNP may act through different mechanisms, including (i) oxidative stress, (ii) carrying exogenous compounds, and (iii) direct effects of translocated NP in the circulation. The purpose of our ongoing studies is to evaluate the mechanism and effects of translocation of nanoparticles across the lung into the systemic circulation (e.g Bagate et al, 2004, 2005). In earlier work we demonstrated that modulation of epithelial and endothelial permeability using $H_2O_2$ or histamine was a prerequisite to enable translocation of NP (Meiring et al, 2005). The objectives of this study were to set-up a more physiological model with PMN influx in IPL and use this to evaluate if PMN influx through the vascular facilitates the translocation of $^{192}$Ir-UFP particles. A particular challenge was to see whether inflammatory activation of neutrophils in the perfusion medium was able to increase the translocation rate compared to our previous study with intratracheal $H_2O_2$ (Meiring et al, 2005). The CFSE labeled PMN provided a good tool to establish the model allowing visualisation of PMN in lung tissue and study activation by measuring markers such as MPO in the perfusate. However, as compared to our previous studies no translocation of $^{192}$Ir-UFP (18 nm) was detected in normal isolated perfused lungs. Also no translocation was observed in lungs pre-treated with histamine on the endothelial side (1 µM) or $H_2O_2$ (0.5 mM) on the luminal side. This was the same for PMN treated groups along with histamine or fMLP. This data is in contrast with our previous experiments (Chapter **2**) in a negative pressure ventilation lung, where we found significant translocation of Ir particles after pre-treatment with histamine and $H_2O_2$. However we consider this model suitable for testing further inflammatory responses mediated by PMN's in the lung. We thus established a model for inflammatory research in an isolated perfused rat lung.

Isolated human PMN's have been used in studies with IPL to evaluate the effect of ischemia/reperfusion injury in the lungs (Lu et al, 1997), as well as in studies where the accumulations of PMNs were investigated in relation to edema (Guidot et al, 1996). This model has a great potential and can provide a wealth of information regarding the interaction of PMN's with the lung. It was from the onset important to evaluate if the PMNs are stable whether labelled or unlabelled with CFSE. The results showed that the PMNs are stable and viable for the duration of the experimental procedure (37 $^{0}$C) if kept on ice before. The

labeling of PM's also allowed visualising the PMN's after entrapment in the lung. Quantification however appeared to be problematic as the lung structure caused considerable autofluorescence. Potential non-specific neutrophil entrapment such as neutrophil attachment to tubing was evaluated by running medium through the tubing the same amount of time as experimental setup. Little to no attachment of PMN was observed in this phantom model. Measuring MPO activity allowed us to evaluate the activation status of PMN in the lung perfusion under conditions used. This analysis showed no detectable MPO in control lungs. MPO activation did not interfere with ACE release. The iridium particles along with PMN did not result in significant translocation of the particles into the perfusate even after MPO levels was higher compared to controls. The same resulted in the groups where the iridium particles with PMN were perfused along with fMLP or histamine. These results are in contrast with our previous findings (chapter 2) where the radioactive particles at least with histamine result in a small but significant translocation.

It was anticipated that neutrophil activation would result in endothelial damage due to $H_2O_2$ release in the lung. However, we did neither measure $H_2O_2$ in the perfusate, nor detect endothelial damage through increased ACE-levels. Also lungs did not have visible oedema after perfusion with (activated) PMN in the perfusate. PMN activation in IPL can cause weight gain and oedema formation (McDonald et al 1987). We established a experimental model for labeled human neutrophils to be used in a rat lung perfusion. Oxidative burst for PMN's at cold temperature were stable after 3 hours compare to 37 $^0$C PMN's. No significant differences in migration were between the labeled PMNs and the activators dose. Once PMN was activated in lung, they stay activated throughout experimental time. The PMN's were not fully activated after lyses of perfused samples. This was visible in experimental condition where fMLP was perfused along PMN's. The metabolic as well as their migration or influx behaviour of PMN's could be fully investigated by this model. Studies like PMN localisation and influx can be measured or quantify with more specialised methods in this isolated lung method. The dye had no effect on the PMN and further acute lung injury parameters can be investigated with this model. It would be our goal to investigate the PMN effects in the negative pressure model.

Several *ex-vivo* treatments of the isolated lungs with either $H_2O_2$, histamine, isolated human neutrophils in the absence or presence of N-formyl-Met-Leu-Phenylalanine (fMLP) were applied. Our objective with these different treatments was to mimic certain physiological states that accompany adverse effects due to particle inhalation. Using $H_2O_2$ we anticipated inducing an oxidative stress, which is also caused by PM inhalation in the lung both by direct radical formation by PM constituents and indirectly by recruited inflammatory cells (review:

Knaapen et al 2004). Oxidative stress has been forwarded as a central hypothetical mechanism in the adverse effects of PM, including ultrafine particles (Donaldson et al, 2003). Earlier on Donaldson et al, (2001) forwarded that oxidative stress and depletion of GSH can affect lung permeability allowing for greater particle passage via lung epithelium into the interstitium. This concept is supported by our previous experiments in the same system using a high concentration of $H_2O_2$ (5 mM) by bolus injection into the lung, to reach a final concentration of 0.5 mM (Meiring et al 2005). In this model we used neutrophil recruitment and activation as a physiological source of ROS and inflammatory mediators. Neutrophil recruitment is characterised by 3 steps namely adherence, extravasation and migration (Sibille and Merchandise, 1993; Strieter and Kunkel, 1994). Inflammatory mediators are also thought to induce vascular leakage or causing gaps between endothelial cells that result in crossing of blood elements or plasma proteins across the endothelium. This increase endothelial permeability helps to facilitate blood elements to sites of injury (Tiruppathi et al 2002). Although this model does certainly not meet all conditions of an inflammatory response, similar models have been applied in other ex-vivo permeability studies (Habib & Clements, 1995; Hulsmann et al, 1996). In isolated perfused rat lungs, a low concentration of $H_2O_2$ (0.25 mM) in the perfusate was shown to increase capillary permeability in the absence of lipid peroxidation (Habib & Clements, 1995). A short-term treatment with $H_2O_2$ (100mM) on the epithelium of human airway tubes caused a sixfold increase in translocation of $^{111}$In-DTPA, which was explained by the opening of paracellular pathways (Hulsmann et al, 1996).

However, much to our surprise even activated PMN in the perfusate did not facilitate particle translocation. This finding adds up to the set of conflicting toxicological data available that particles are transfer from the lungs to blood circulation (Nemmar et al, 2001, Kreyling et al, 2002, Oberdorster et al, 2004) and also from the blood to the lung lumen (Heckel et al, 2004). All these studies made use of in vivo models. The translocation was small in most cases, which might suggest that particle translocation is impeded by the large set of barriers in the lung. Upon deposition in the pulmonary system particles encounter surfactant, macrophages, and have to cross the epithelial barrier, the interstium and the endothelial layer to reach the blood capillaries (Review Agu et al, 2001).

In comparison to our previous studies in IPL (**Chapter 2**), we modified several conditions. First, we used a modulation of oxidative stress that is more close to reality and used activated neutrophil influx in the lung. Secondly, we used positive pressure ventilation instead of negative pressure ventilation. A main reason for this change was to minimise oedema formation which is a huge problem in negative pressure ventilation. A few hyper ventilation sessions were done to prevent the capillaries collapsing. Another variable was the

medium where we made use of Krebs-Henseleit buffer substituted with Hepes and Albumin. We demonstrated that the neutrophils are activated by release of MPO, ROS and active chemotaxis (*in vitro*). In this study, $^{192}$Ir-UFPs were instilled from a freshly prepared suspension of aerosol led into water. Although instillation uses a similar dose, the dose rate is much higher and which usually leads to a more rapid influx of inflammatory cells (Oberdorster, 2001). Nevertheless, the installation of 80 µg $^{192}$Ir-UFP did not lead to increase activation of PMN or translocation as previously observed with a bolus-injection of $H_2O_2$ (Meiring et al, 2005). On the other hand the positive control used in this study (1 µM histamine, perfusate) also did not reveal translocation of $^{192}$Ir-UFP. This suggests that the change to positive pressure ventilation is the cause of our incongruent data. The instillation of particles might also contribute to the fact we didn't observed any translocation this time. Ultrafine particles might form conglomerates while in suspension and thus might hinder translocation of $^{192}$Ir-UFP to the systemic circulation. Our previous study made use of inhalation exposure over 2 hours, which might facilitate translocation as the dose rate over time is far less than with the instillation procedure. Clearly more work is needed to address this issue.

The mediator release of ALP, ACE and proteins are not revealing any clear picture. We anticipated that hydrogen peroxide would cause epithelial damage that might facilitate translocation as suggested by our previous study (Meiring et al, 2005). Usatyuk and colleagues (2003) suggested that reactive oxygen species might affect endothelial permeability. Another mechanistic is that the possibility of endothelial permeability via inflammatory process might cause particle translocation from the lumen side. The neutrophilic influx along with fMLP addition didn't cause any significant effect on the translocation.

In conclusion the translocation of ultrafine particle seems to be not possible due to our positive pressure **IPL** experimental model. The affects of intratracheal instillation along with positive pressure perfusion model is not suitable to answer the observed epidemiological observation. We did however establish a suitable *ex vivo* lung inflammatory model for further pulmonary research. There should be a need to further investigate the physiological and histological effects of particles in this model.

Figure legends

**Figure 1** Effect of pre-loading of neurophils on their function as measured by spontaneous or PMA induced oxidative burst The opening ring group represents the spontaneous (room temperature) PMN group. PMA stimulated ROS production is affected at concentrations over 0.3 uM CFSE. Data are mean and standard deviations.

**Figure 2** Represents the change in neutrophil activation over time with the various activators. Drastical change in neutrophil activation over time (30 min-130 min) in the warm temperature stored neutrophils ($37^0$C). The oxidative burst (Release of reactive oxygen species (ROS) are as well very low in these groups after 130 minutes. PMA activation also didn't activate this PMA as the the average is the same as for the spontaneous group. Neutrophil activation however is sensitive in the PMA treated group cold storage neutrophil. The average between the groups with time does not change much. Bars present from left to right with the blocks the timepoins. Data are mean of 2 experiments with PMN's from 3 subjects.

**Figure 3** Myeloperoxidase (MPO) release in control and PMN perfused rat lung during a 90 minute perfusion interval. PMNs were added to the lung perfusate after 15 min equilibration The graph represents the activation of human neutrophils after contact with rat lung. The data are expressed as mean ± S.E.M. Data of 3-4 experiments

**Figure 4A** Myeloperoxidase (MPO) release from isolated perfused rat lung. Graph with table represents with the individual perfusions with treatments. Highlited area compare the MPO release from the individual groups.

**Figure 4B** Myeloperoxidase (MPO) release from isolated lung perfusion (IPL) perfusate during the 2 hours perfusion. The groups represent the different treatments. The data are expressed as mean ± S.E.M.

**Figure 5** Radioactivity of pooled perfusate samples after intracheal installation of 192Ir-UFP and concomitant perfusion with histamine (positive control), and combinations of PMN with or

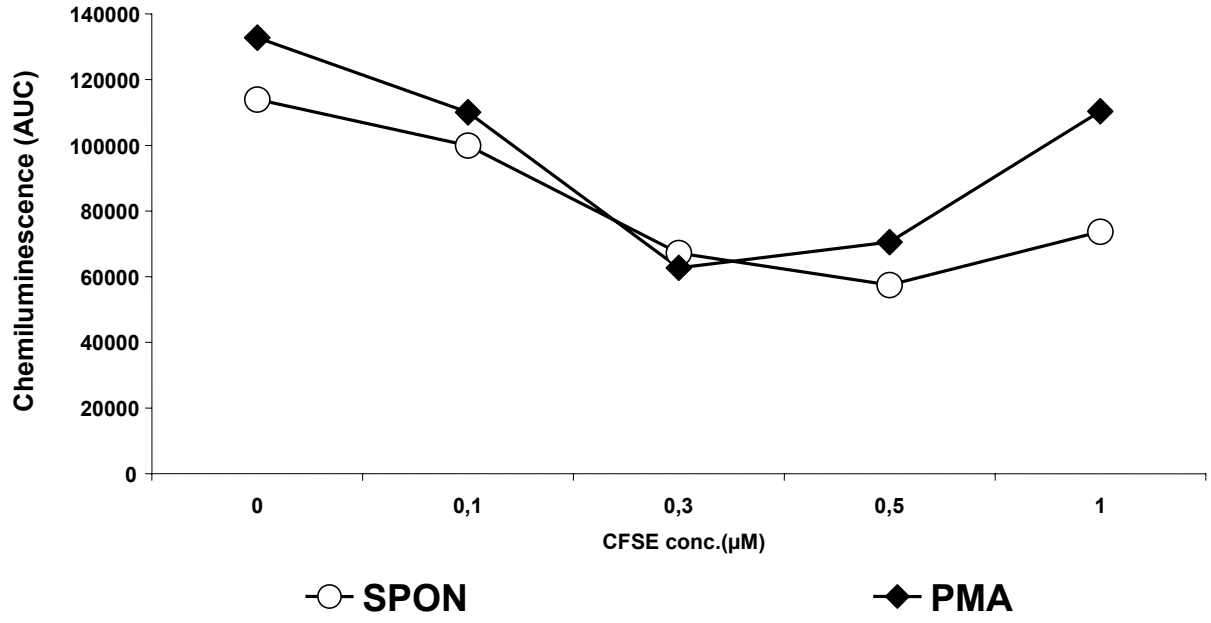
without activation with fMLP. The data show no translocation of UFP as compared to the 192Ir-UFP alone.

**Figure 6 A, B, C** Fluorescence picture taken from fixed lung perfused with labelled CFSE neutrophils Influx of CFSE-loaded PMN into lung tissue. The CFSE dye clearly demonstrates that neutrophils gather and remain in the lung after been perfuse through the lung. Typical localization are along the alveolar septa.

***Table 1*** Experimental protocol for the isolated perfused lungs. All perfusions were done under positive pressure ventilation. Following a 15 minute period of equilibration, during which the lungs were already ventilated and perfused, the experiment started by the instillation of freshly produced $^{192}$Ir-UFP. The perfusate was collected continuously and sampled at 15 minutes intervals. Radioactive particles instilled at time-point 15 minutes except controls. The starting PMN concentration in the perfusion medium was $1\times10^6$ cells/ml.

| Treatment | saline | PMN | $^{192}$Ir-UFP | FMLP | Hist(it) | Hist(perf) |
|---|---|---|---|---|---|---|
| 1 | + | - | - | - | - | + |
| 2 | - | - | + | - | + | - |
| 3 | - | - | + | - | - | - |
| 4 | - | + | + | - | - | - |
| 5 | - | + | + | + | - | - |
| 6 | - | + | + | - | - | + |
| | | | | | | |

**Figure 1**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120000
100000
80000
60000
40000
20000
0
0
0,1
0,3
0,5
1
CFSE conc.(µM)
SPON
PMA

**Figure 2 .**

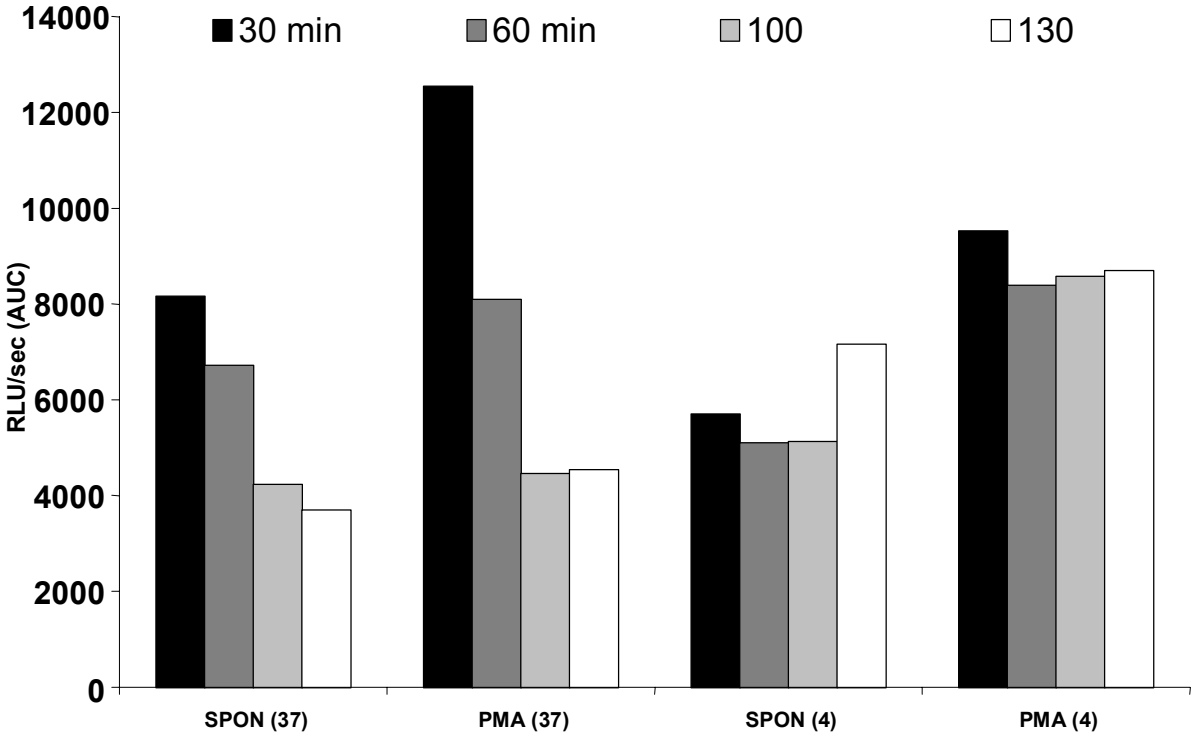
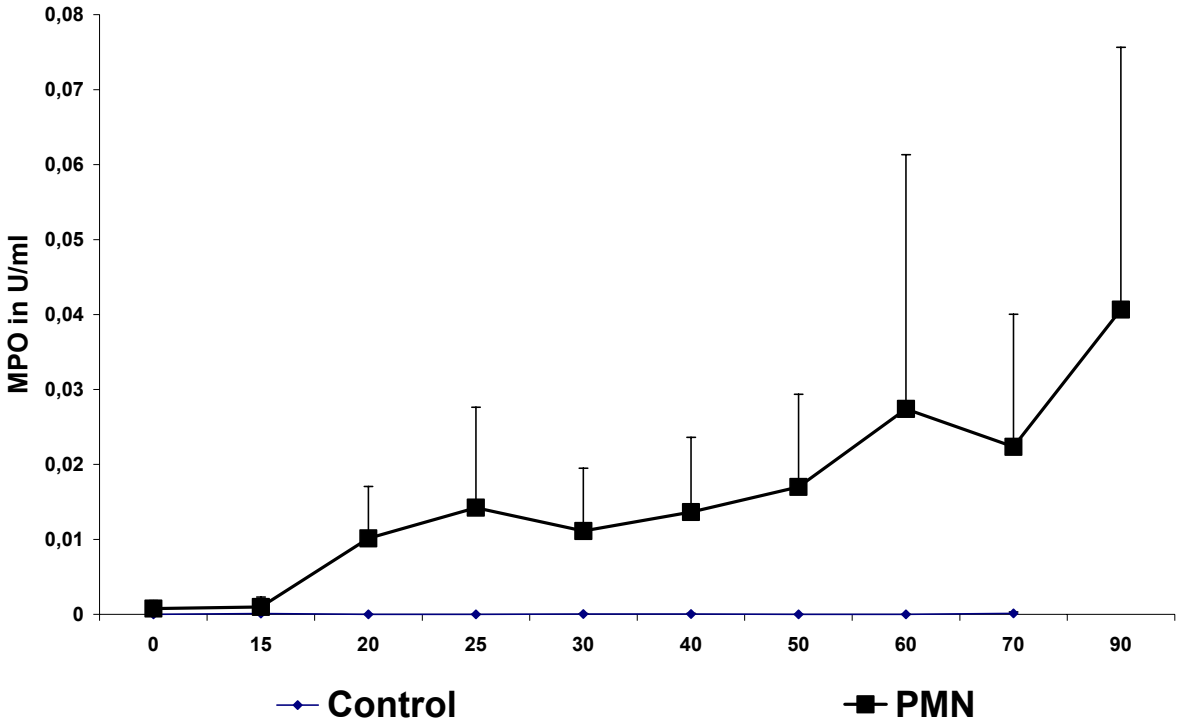

14000
12000
10000
8000
6000
4000
2000
0
RLU/sec (AUC)
30 min
60 min
100
130
SPON (37)
PMA (37)
SPON (4)
PMA (4)

**Figure 3**


MPO in U/ml
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0,07
0,06
0,05
0,04
0,03
0,02
0,01
0
0
15
20
25
30
40
50
60
70
90
Control
PMN

**Figure 4 A**

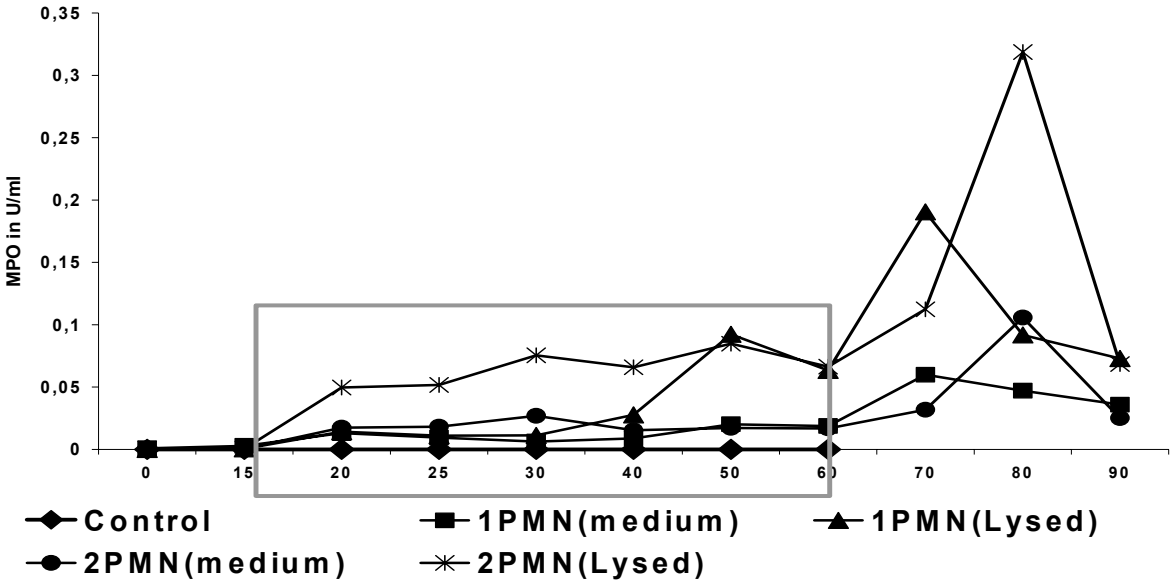



**Figure 4 B**

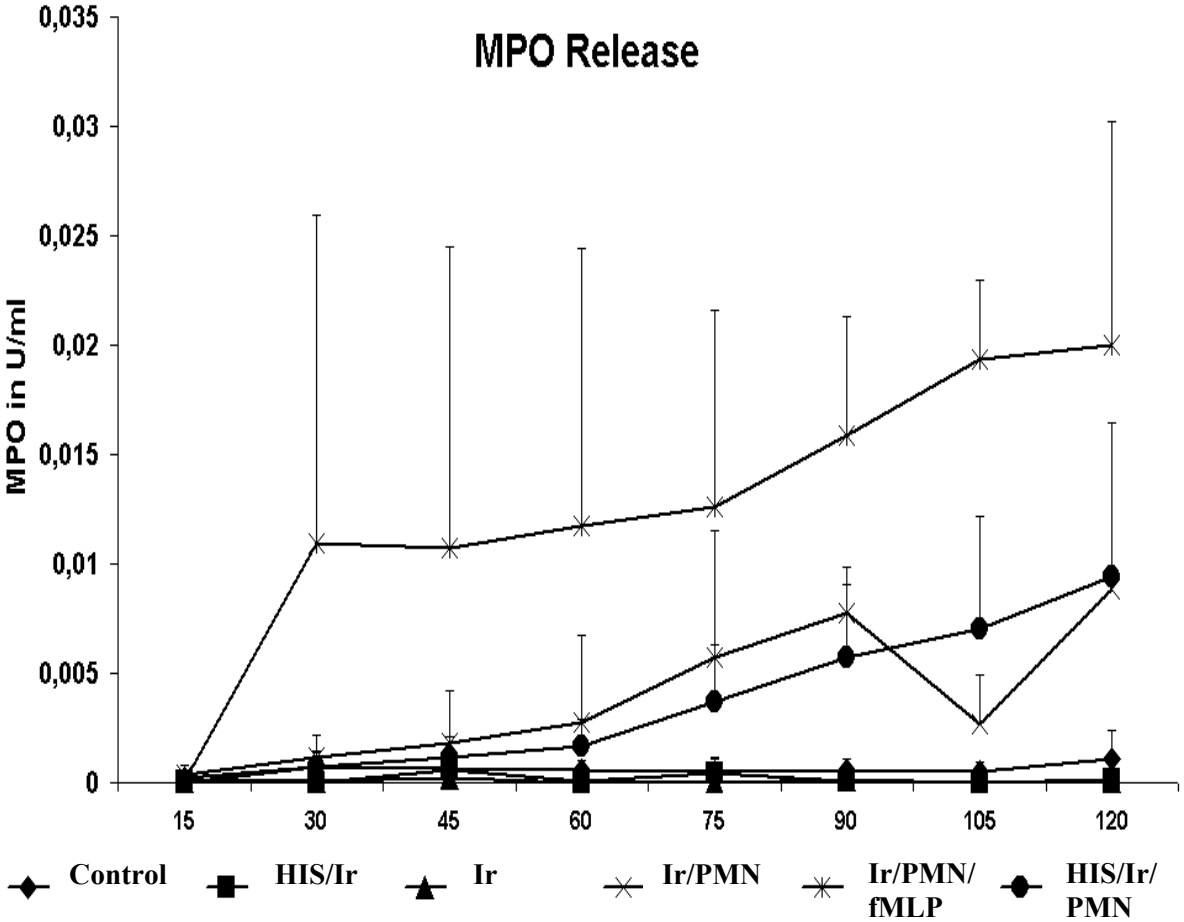
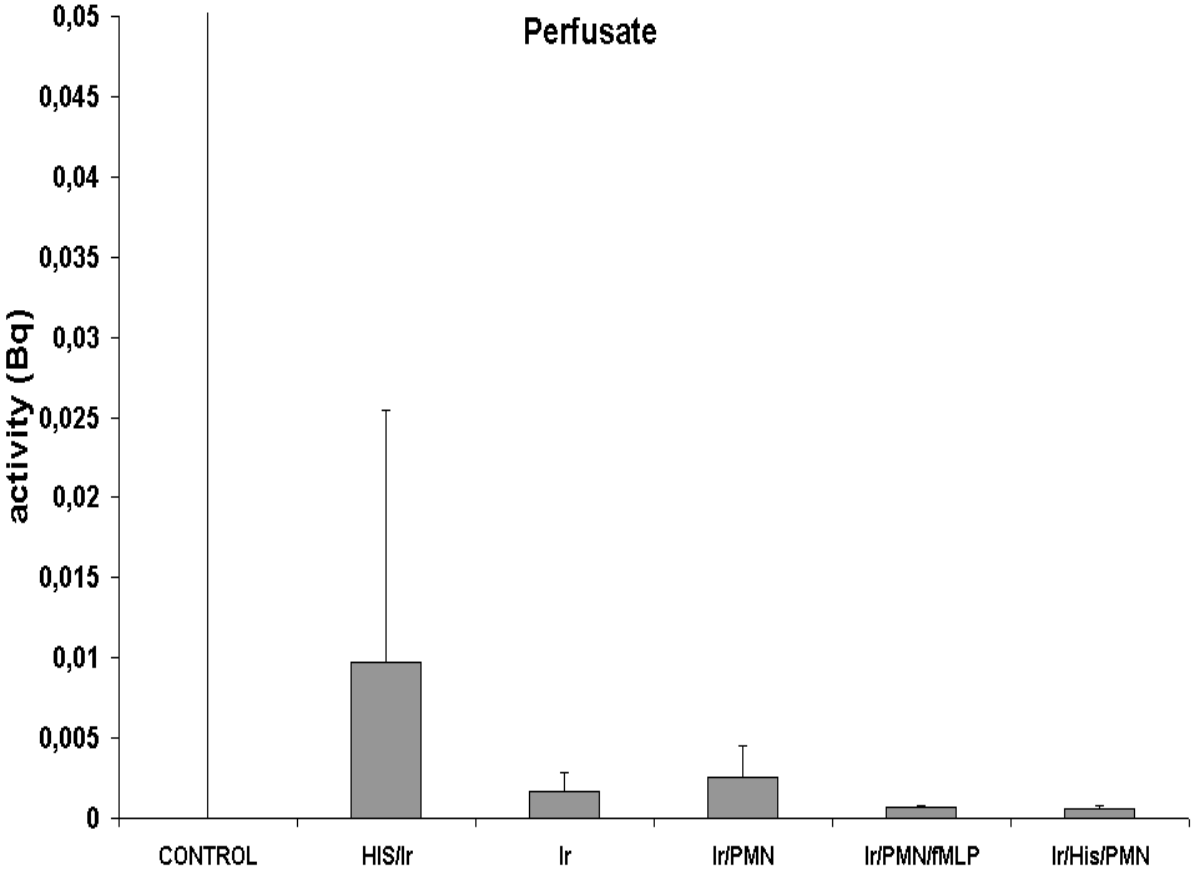


**Figure 5**


Perfusate
activity (Bq)
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0,045
0,04
0,035
0,03
0,025
0,02
0,015
0,01
0,005
0
CONTROL
HIS/Ir
Ir
Ir/PMN
Ir/PMN/fMLP
Ir/His/PMN

**Figure 6 a**

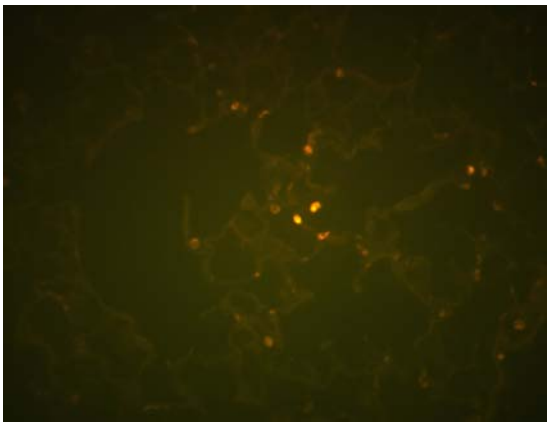


**Figure 6 b**

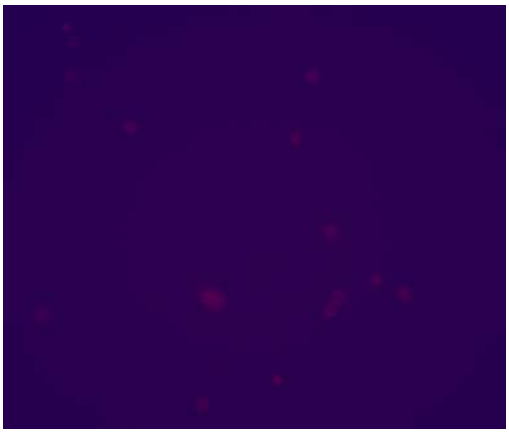
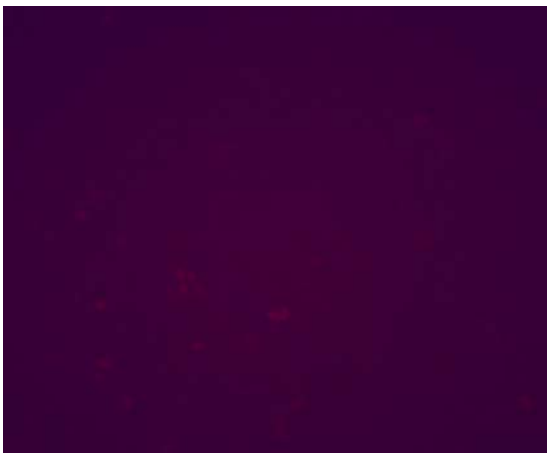


**Figure 6 c**



# Chapter 4

# AMBIENT PARTICULATE MATTER AFFECTS CARDIAC RECOVERY IN A LANGENDORFF ISCHAEMIA MODEL

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**ABSTRACT**

Exposure to ambient particulate matter (PM) is associated with increased mortality and morbidity among subjects with cardiovascular impairment. We hypothesised that exposure of spontaneously hypertensive (SH) rats to PM impairs the recovery of cardiovascular performance after coronary occlusion and reperfusion-ischemia. SH rats were exposed by intratracheal instillation to saline, standard urban PM (Ottawa-dust EHC-93, 10 mg/kg of body weight) or endotoxin (LPS, 350 EU/animal) to induce a similar pulmonary inflammation. At 4 h post-exposure, hearts were isolated and retrograde perfused in a Langendorff model. The experimental protocol included 35 minutes coronary occlusion followed by 120 minutes reperfusion during which left ventricular developing pressure (LDVP), coronary flow (CF) and heart rate (HR) were measured. Baseline LVDP in particle-instilled SH rats was significantly decreased compared to saline-instilled animals. In addition, after ischemia the recovery of LDVP was much slower in rats pre-treated with PM or LPS compared to saline instilled rats. The direct effects of the soluble PM fraction and the role of $Zn^{++}$ were also tested cardiomyocytes (H9C2 cells). Both particle-free filtrate and $Zn^{++}$ inhibited ATP or ionophor-stimulated calcium influx in cardiomyocytes. This inhibitory effect was related to an effect on calcium channels as shown with Nifedipine. This study provides evidence that exposure to instillation of model PM has reversible acute effects on the recovery of cardiac physiological parameters after ischemia. The effect may be caused by a direct action of soluble metals on calcium homeostasis in heart, but pulmonary inflammation may also play a significant role.

Keywords:
Particulate matter, ischemia, recovery, calcium influx

## Introduction

Epidemiological studies have demonstrated a relationship between exposure to ambient particulate matter (PM) and adverse effects in sub-populations with cardiopulmonary diseases. A positive correlation between increase in air pollution and cardiopulmonary-related morbidity and mortality has been reported in various studies (Pope et al, 2004; Dockery et al, 1993 ; Peters et al, 2004). Either direct and indirect effects of particles or their constituents have been suggested to explain this effect. Experimental and panel studies have indicated that PM can cause a direct increase in systolic blood pressure and altered autonomic nervous system control of cardiac rhythm (Ibald-Mulli et al, 2001), leading to change in heart rate variability (Godleski et al, 2000; Pope et al, 1999; Gold et al, 2000) and increase in heart rate (Peters et al., 1999) and an increased risk for myocardial insult within two hours after exposure (Peters et al, 2004).

Seaton et al, (1995) were the first to suggest an indirect effect through pulmonary inflammation caused by particle deposition leading to increased blood coagulability and plasma viscosity, thus indirectly promoting myocardial infarction. Such indirect effects may be mediated by the release of cytokines such as interleukin (IL)-1 and IL-6 from the lung into the systemic circulation (Van Eden et al, 2001). Residual oil fly ashes (ROFA) have been reported to induce COX-2 expression and release of $PGE_2$ from lung epithelial cells (Samet et al, 2000). PM also caused the release of endothelin from the lung to the blood (Bouthilier et al, 1998). Endothelin is well known to be involved in congestive heart failure, hypertension, vascular remodelling from arteriosclerosis, post-angioplastic restenosis, and in asthma (Vincent et al, 2001). In addition, histamine has been shown to mediate diesel particle induced peripheral thrombosis via $H_1$ receptor- activation since a specific antagonist was able to block this effect (Nemmar et al, 2003). We recently demonstrated in isolated perfused lung that the translocation of ultrafine particles is enhanced upon administration of histamine on the endothelial side or hydrogen peroxide on the luminal side (Meiring et al, 2005).

However, much less is known about the mechanisms by which PM and its constituents affect cardiovascular performance. A disturbance of vascular function elicited by PM has been reported by our group (Bagate et al, 2004a,b ; Knaapen et al, 2001) as well as others (Batalha et al, 2002; Brook et al, 2002; Mills et al, 2005; Nurkiewicz et al, 2006). This suggests that if sufficient particles or their soluble constituents are translocated upon inhalation and reach the blood they are capable to induce direct effects on the cardiovascular system. In-vitro studies with diesel exhaust particles (DEP) showed negative inotropic and positive chronotropic effects followed by cardiac arrest (Sakakibara et al, 1994) in guinea pig isolated cardiac muscle preparations. Intravenous administration of DEP in guinea pigs induced arrhythmia and death by atrioventricular block (Minamia et al, 1999). We

hypothesised that exposure to PM, apart from exerting a direct effect on the lung and heart, could also impair recovery of of the heart after ischemic insult. Such an effect would only become visible after substantial damage such as after coronary occlusion and reperfusion-ischemia as seen in survivors of myocardial infarction. In other words, the effects of PM on mortality may not be caused by causing MI but by a slower recovery from small or larger myocardial infarctions. To test this hypothesis we subjected isolated hearts from SH rats, after in vivo exposure to PM, to ischemia by coronary occlusion and studied functional recovery and pathological damage during reperfusion. Ischemia/reperfusion insult has been extensively studied for its clinical relevance and the Langendorff model with the retrograde perfusion is the standard ex-vivo technique that is used for experimental work (e.g. Xu et al, 2003). To elucidate the role of pulmonary inflammation in the effect of PM, SH rats were treated by intratracheal instillation with standard urban PM 10 (EHC-93) and compared to endotoxin (LPS) and saline-instilled animals. At 4 h post-exposure, hearts were isolated and retrograde perfused in the Langendorff model. The central aim of our study was to investigate the effect of PM exposure on physiological performance of the heart in an ischemic/reperfusion model.

## Methods

### Animals

Male spontaneously hypertensive rats (SHR/NHsd) of 11-12 weeks old and weighing 250-350 g were obtained from the breeding colony of Harlan (Indianapolis, Indiana, USA). Immediately after arrival, the animals were weighed and randomly allocated. Animals were housed in macrolon cages Type 3, in a room at constant climate with a 12 h light/dark cycle (light on at 8:00 a.m.) and allowed free access to an automatic drinking water system and SSP-Tox standard food (Hope Farms, Woerden, The Netherlands). Basic inspection of animals took place every day. The Ethical Review Committee of the National Institute for Public Health and the Environment (RIVM) approved experiments. The animals were instilled with particles (EHC-93, 10 mg/kg of body weight in a concentration of 5 mg/ml), LPS (350 endotoxin units/animal, 0.5 ml) or saline (2ml/kg of body weight) through a cannula inserted into the trachea just above the bifurcation. Before this manipulation animals were briefly anaesthetised by 4% halothane inhalation.

*Chemicals and particles*

Lipopolyssacharide (LPS) was obtained from (Sigma-Aldrich Chemie Gmbh), Ketamine (Aesculap , Boxtel, The Netherlands) ; Rompun (Bayer AG, Leverkusen) ; PM (EHC-93) (Health Effects Institute, Ottawa, Canada). All other chemicals were of pro-analysis quality

from Merck (Darmstadt, Germany). Compounds were dissolved in double distilled water and freshly prepared with saline before each experiment. For in vivo instillations we used urban PM (EHC-93) that was collected in Ottawa (Canada) in 1993. LPS content of EHC-93 was determined using the Limobulus Amoebocyte Lysate (LAL) assay as described previously (Schins et al., 2004). A suspension of EHC-93 particles was obtained by suspending EHC-93 in saline at the desired concentration by sonication.

For details on particle size and composition we refer to previous publications (Bagate et al., 2004a). For in vitro perfusions we used zinc sulphate, since zinc is the major soluble metal in the PMsample that was used. All the *in vitro* experiments were done with the soluble fraction of EHC-93 or solutions of metals. The soluble fraction was prepared from the EHC-93 suspension, by filtering with a syringe mounted on a 0.1-µm filter (Minisart RC15, Sartorius AG, Göttingen-Germany). This procedure was shown not to affect the concentration of active soluble metals such as zinc, copper, magnesium and vanadium in the filtrates (data not shown).

**Isolation of hearts and experimental protocol**

Necropsy took place at 4 h after instillation of PM, LPS or saline. Animals were anaesthetised with an i.p. injection (1ml/kg of body weight) of a mixture of ketamine (100 mg/ml) and rompun (20 mg/ml) in a ratio of 10:4. A pilot study comparing the effect of this procedure to rats receiving no , but killed by decapitation, showed no significant effect on the heart function (data not shown). After opening the thorax, a syringe containing a cold Krebs-Ringer (KR) solution (4 °C) was inserted in the aorta through a canula. The heart was rapidly excised and immediately suspended for retrograde perfusion (Langendorff model) with pre-warmed (37 °C) and oxygenated (95% $O_2$ / 5% $CO_2$) modified KR solution at 60 mmHg constant pressure with the following composition (mM) : NaCl 118 ; KCl 5.9 ; $CaCl_2$ 2.5 ; $MgSO_4$ 1.2 ; $NaH_2PO_4$ 1.2 ; $NaHCO_3$ 24.9 ; glucose 11.1, pH was adjusted at 7.4. The Left ventricular Developed Pressure (LVDP) was measured via a water filled balloon inserted into the left ventricle and connected via a catheter with a disposable transducer (Spectramed Becton-Dickinson). LVDP and heart rate (HR) were recorded on a Graphtec Linear Recorder Mark 8, Type WR 3500 (Hugo Sacks Electronic Germany) equipped with differentiator amplifiers (DC Bridge amplifier DBA 660 , Hugo Sachs Electronic Germany) and a heart rate module (HRM 669 Hugo Sachs Electronic, Germany). The dP/dt max (velocity of contraction) was calculated from the recorded parameters. Diastolic ventricular pressure was adjusted between 5 and 10 mmHg. Coronary flow was established by measuring the volume of perfusate leaving the coronary system. Basal flow was between 8 and 10 ml/min. The experimental procedure for ischemia and recovery was established as follows. First, all the hearts were equilibrated for 30 min. An ischemic period of 35 min was induced by coronary artery occlusion. Subsequently a reperfusion period of 120 min was allowed for all groups.

Other experiments on isolated hearts were done without previous ischemia and done on hearts of SH rats pre-treated with saline. In these experiments the water-soluble fraction of PM or a solution of $Zn^{++}$ ,as major metal present in our particles preparations, were tested by a 10 min infusion. A similar equilibration period for isolated hearts was applied before infusion of test-solutions.

## Pathological examination of the hearts after perfusion

Haematoxylin-eosin 5 stained sections (µm) were evaluated using fluorescence microscopy (Olympus BX40, filter set WIB) to identify early ischemic versus normal regions of the heart by their autofluorescence pattern (Osornio and Ossa, 1993). Autofluorescence was evaluated in blind manner without knowing the experimental conditions that the rats were subjected to. Ischemic regions were reported for the right and left ventricle (free wall and septum) accordingly to subendocardial, subepicardial or transmural location. The extension of the ischemic areas was estimated as a percentage of the histological section affected and reported as: 0 = no ischemia; ± = < 5%; + = 6 – 20%; ++ = 21 – 35%; +++ = 36 -50%; ++++ = > 50%.

*Calcium imaging in cardiomyocytes.*

Cardiomyocyte H9c2 cells were obtained from European cell collection (EACC). Cells were cultured in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin, and 1 mM pyruvate in humidified air (CO2 5%) at 37 °C. For imaging experiments, cells were plated onto poly-D-lysine-coated glass coverslips. Cells were grown for 3–5 days sub-confluent cultures. Prior to loading with fluorescent dyes, cells were preincubated for 30 min with medium (2% bovine serum albumin/ extracellular medium) consisting of 121 mM NaCl, 5 mM NaHCO3 ,10 mM Na-HEPES, 4.7 mM KCl, 1.2 mM KH2 PO4 , 1.2 mM MgSO4 ,2 mM CaCl2 ,10 mM glucose, and 2% bovine serum albumin, pH 7.4 at 37°C. (Pacher et al., 2000, 2001 ; Szalai et al., 2000).To measure intracellular calcium $[Ca]_i$, cells grown on glass cover slips were washed twice with Hanks Balanced Salt Solution (HBSS) and incubated in HBSS, pH 7.3 containing 1 µM Fura-2/AM for 30 min at 37 °C. Following incubation the cells were washed twice with 4 ml Fura-2/AM free HBSS and mounted on an inverted microscope (Zeiss Axiovert, Germany) and washed for another 5 min to remove excess of Fura-2/AM. Fluorescence of 6-8 cells was simultaneously monitored using digital imaging fluorescence microscopy (Photon Technology International, New Jersey, USA) as described earlier (Bickmeyer et al., 1998). Briefly, the images were obtained through an oil immersion objective (40 x) and visualized with a high sensitivity camera (SIM-ICCD-04). Fluorescence ratios were obtained from the two

background corrected images at 340 and 380 nm excitation and 510 nm emission wavelengths. In calibration experiments we used HBSS containing 10 μM ionomycin and 10 μM gramicidin in the presence of 10 mM $CaCl_2$ (R-max) and without calcium but with 1 mM EGTA (R-min). Cell auto-fluorescence was determined in 10 mM manganese chloride.
Experiments were carried out with the soluble fraction of PM or $Zn^{++}$ solutions evaluating at least seven to twelve different cells per run, and a total of 35-100 cells per experiment. For calcium influx, cells were challenged with a depolarisation solution KCl (50 mM) or receptor-dependent calcium influx were established with ATP (100 μM ). Solutions were tested in two periods separated by 30 min interval. Particle-free filtrate (1-50-100 μg/ml final concentration) or $ZnSO_4$ (50 μM) were applied during the 30 min interval in order to verify their ability to modify calcium influx in cardiomyocytes. Cytotoxicity testing (MTT-assay) revealed that neither PM nor Zn at at the applied concentrations led to cell death (data not shown). The effect of PM soluble components on intra-cellular calcium influx was evaluated with thapsigargin (1 μM) whereas their effect on extra-cellular calcium influx was tested with the calcium channel agonist, the dihydropyridine Bayk 8344 (1-5-10 μM). The results of each individual experiment were evaluated as the mean variation of the ratio 340-380, depicting the emissions at 510 nm using 340 or 380 nm excitation.

***Statistical analysis***

**Results are expressed as means ± S.E.M. Differences were tested for statistical significance by the paired or unpaired Student's t-test and one-way or two-way variance analysis on repeated measurements when appropriate. Multiple comparisons between groups were performed by Tukey's test or Newman Keuls test. A P value less than 0.05 was considered significant. All statistics were run with SPSS 9.0 for Windows.**

**RESULTS**

Effect of pre-treatment on baseline parameters of isolated perfused heart.

The baseline performance of hearts taken from SH rats after exposure to PM or LPS in comparison to saline is shown in Table 1. The isolated hearts were allowed stabilization for 30 min beforestarting the recording of functional parameters. After PM exposure a slight increase of baseline coronary flow (CF) and heart rate (HR) was noted. In contrast a significant decrease of LVDP was observed in SH rats 4 h after instillation. LPS also elicited a decrease of LVDP but this change was not statistically significant. No pathological abnormalities were found in hearts up to 120 min of baseline perfusion (data not shown).

Effect of pre-treatment and ischemia on cardiac function

After the stabilization period, isolated hearts were subjected to ischemia during 35 minutes by arterial occlusion, and subsequent release of occlusion and normal retrograde perfusion during 120 minutes. The physiological parameters were monitored during the whole interval.A significant difference in LVDP-recovery was observed between the saline-treated rats and the rats exposed to PM or LPS (Fig. 1). In control animals the an initial decrease to 50% of baseline value and restoration to 90% of the baseline value within 60 min after release was seen. After that, LDVP remained more or less constant during the rest of the perfusion period. When SH rats were pre-treated with PM or LPS the isolated heart had a clearly reduced ability to recover to baseline levels after occlusion in comparison with the saline treated animals (Fig. 1). LVDP was down to 50 mm Hg in both cases after occlusion and remained at this level until the end of the reperfusion period. No significant effects of occlusion or exposure to PM and LPS were observed for heart rate, which remained constant at all time points and treatments (Table 1). A totally different response was noted for coronary flow. After releasing the occlusion, CF goes back to baseline values. Subsequently both in saline and LPS treated rats a gradual decrease in CF was noted during the reperfusion period. On the other hand isolated hearts from PM exposed SH rats showed complete restoration of CF and no gradual decrease at all (Table 1)**.** To test whether soluble metals are able to cause some effects on cardiac performance as observed after in-vivo PM pre-treatment, a Zn-containing solution was perfused in the heart through the perfusion solution at 0.1 ml/min to reach a final concentration of 10 µM within 10 min (Fig. 3). After that $Zn^{++}$-free solution was added to the system, which allowed elimination of $Zn^{++}$ from the perfusion chamber. The infusion of $Zn^{++}$ elicited a rapid decrease of LVDP and heart rate (Fig. 3a,b). The dP/dt max, calculated from the previous parameters was also significantly reduced (data not shown). The impairment of cardiac function measured by LVDP and heart rate started immediately upon $Zn^{++}$ infusion and remains the same during the whole perfusion period, even though no $Zn^{++}$ was present in the perfusate (Fig. 3a).

Histopathology

Histopathological evaluation of ischemic damage was done at the end of perfusions in a number of hearts and may indicate the differences induced by exposure accumulated with damage induced by ischemia/reperfusion. No differences in mean severity index (0 = no ischaemia; 1 = < 5%; 2 = 6 – 20%; 3 = 21 – 35%; 4 = 36 -50%; 5 > 50%.) were seen between saline treated (SI= 2.33 ± 1.89) and SH rats exposed to PM (SI= 2.33 ± 1.53) or LPS (SI= 1.75 ± 1.06). The ischemic damage was most prominent in the left ventricle, more specifically in the free wall than in the septum, but again this was not different between different exposures (Fig. 2).

**Effect of PM filtrate on $Ca^{++}$ uptake in cardiomyocytes**

To test whether the effects of in vivo pre-treatment on cardiac function were due to a direct effect of soluble components on the heart muscle, as noted previously for aorta (Bagate et al, 2004a,b) we studied Ca-uptake in isolated cardiomyocytes. Isolated cardiomyocytes were loaded with the fura-2, and uptake of Ca was studied after stimulation with KCl (receptor-independent) or with ATP (receptor-dependent). Repetitive (30 min intervals) treatment with KCl (50 µM)or ATP (100µM) led to a reproducible increase in intracellular calcium concentration mainly from the extracellular compartment. (Fig. 4 a and b).. Addition of PM filtrate in concentrations between 1 and 100 µg/ml after the first stimulus caused an steady increase in baseline intracellular $Ca^{++}$ levels (Fig 6), but also a reduction of Ca-influx by the second stimulus (Fig 6). Nifedipine (2 µM), a calcium channel antagonist inhibited the baseline calcium influx elicited by PM (100 µg/ml) (fig. 5) as well as that induced by KCl. As a positive control to this experiment, we used the calcium channel agonist BayK (5 µM) and found that Nifedipine (2 µM also inhibited BayK induced Ca-uptake in cardiomyocytes (data not shown). The calcium influx upon second stimulation with KCl or ATP was completely abolished by $Zn^{++}$ at 50 µM ($p<0.01$**,** Fig. 6a,b). PM filtrate also caused a decrease of calcium influx by both KCl and ATP. This effect became significant at a PM filtrate concentration of 100 ug/ml($p<0.05$ for ATP and $p<0.01$ for KCl**,** Fig. 6c,d).

In order to establish the mode of action of PM, i.e. on uptake from the extra-cellular compartment or blocking of intracellular release, we performed Ca-imaging experiments with the dihydropiridin agonist Bayk 8344 or thapsigargin as an intracellular Ca-ATPase blocker. Two minutes before challenging the cells with thapsigargin, which normally gives an explosive release of intracellular Ca, the medium was replaced with a calcium-free medium supplemented with 1 µM EGTPA in order to prevent the entrance of extracellular calcium.

BayK (5μM) elicited a calcium influx in cardiomyocytes, which was inhibited by PM filtrate at both 1 and 50 μg/ml. ($p<0.05$ and $p<0.01$ respectively, Fig. 7 left). In contrast, PM filtrate (100 μg/ml) did not inhibit the calcium influx from the intracellular stores elicited by thapsigargin (2 μM) (Fig. 7 right). This shows that calcium channels are affected by components in the PM filtrate and prevent influx from extracellular compartment.

**Discussion**

The purpose of this study was to investigate the effect of PM exposure on cardiac function and recovery after ischemia reperfusion injury in SH rats. Our present data showed that pre-treatment of SH rats with ambient particles or LPS adversely affects the ability of the isolated heart to recover after an ischemic insult caused by coronary occlusion, although no difference in pathological damage was observed at the end of the experiment. The reduced recovery was mainly seen in LDVP and observed at 4 h post-exposure. We showed that soluble components in the PM preparation and Zn as the main soluble metal in the PM also elicited a depression of LVDP. However other metals may have a similar effect but have not been tested. Additional in vitro studies in cardiomyocytes suggest that the disturbance of cardiac function by PM may be caused by an effect on calcium homeostasis by bioavailable metals from PM. However, since LPS induced a strong effect on cardiac recovery an indirect effect through pulmonary inflammation also may play a significant role.

Heart attacks accompany myocardial ischemic damage, which has been observed in epidemiological studies (Peters et al., 2004). Myocardial ischemia has been implicated in leading to electrical instability and lethal arrhythmia (Ghuran et al, 2001, Brugada, 2001). Myocardial ischemia also leads to loss of cell membrane integrity, which disrupts both depolarization and repolarization leading to cardiac instability that can cause sudden death in the clinical scenario. We suggested that pre-exposure to PM may deteriorate the capacity of the heart to recover after myocardial ischemia and used an ex vivo isolated rat heart model using coronary occlusion on ischemia/reperfusion recovery of hearts from pre-instilled

spontaneously hypertensive rats. SH rats have been used in this study because they are suggested, as a model of human with cardiovascular disease and since this subpopulation is a primary group at risk for effect of ambient PM (Pope et al, 2004). SH rats are a suitable animal model for systemic hypertension and underlying cardiac disease and it has been reported that cardiac effects of PM are stronger in the SH than the Wistar Kyoto rat (Kodavanti et al, 2000; 2002a).

PM consists of several fractions based upon main constituents, for example the organic fraction, including polyaromatic hydrocarbon (PAHs), the inorganic fraction including sulphate, nitrate, ammonium and metals, and the biogenic fraction, which contains essentially bacterial constituents (Dreher et al, 2000). It is unclear which of the PM constituents are involved in the cardiovascular effects seen in epidemiological studies. Intra-tracheal instillation of different PM caused PMN influx in rat lung (Bagate et al, 2004a; Kodavanti et al, 2003) and this inflammation was related to the soluble transition metals (Dreher et al, 1997; Kodavanti et al, 2002b). Although the soluble metals seem to play a major role in the effects observed on the heart, current studies do not allow discrimination between an indirect effect of inflammation and a direct effect of bioavailable metals. At the doses used both PM and LPS instillation elicited similar pulmonary inflammation (Bagate et al, 2004a). The inflammation could induce a release of pro-inflammatory mediators such as endothelins from the lung, which may be released in the blood and cause impairment of cardiovascular function (Prasad et al, 1991; Nurkeiwicz et al, 2006). However our previous work has demonstrated that lung inflammation based on cellular counts was maximum at 24 h post-instillation (Bagate et al, 2004a), at which time-point cardiac effects were no longer present (data not shown). Our previous studies in the same animal model have demonstrated that intra-tracheal instillation of 10 mg/kg PM, plasma concentrations of soluble metals were maximal at 4 h post-instillation (Bagate et al, 2004). Interestingly, the reduced recovery from ischemia was also seen at 4 h but not at 24 h after instillation. These data suggest that soluble metals could be involved in impairment of cardiac function. This time frame also fits with current epidemiological evidence that shows the strongest relationship between onset of myocardial infarction and exposure to traffic at 1 or 2 h after exposure (Peters et al, 2004), although traffic related PM typically contains much less metals. This is an argument to consider the role of the pulmonary inflammation, and is confirmed by the fact that in LPS pretreated rats also a decrease of LDVP was observed at reperfusion.

The role of soluble metals in cardiovascular effects of PM has been indicated by several studies (Kodavanti et al, 2002a,b 2003; Bagate et al, 2004a,b). However, the mechanism by

which PM and its soluble components elicit a disturbance of cardiac function is not well understood. Calcium is known to play a key role in cardiac contraction and we therefore investigated whether PM can induce its cardiac effects through the disturbance of calcium haemostasis. On the one hand, it is still not clear whether the impairment of calcium haemostasis is related to disturbance of intracellular stores or extracellular calcium entrance. The effect of soluble metals on receptor dependent calcium influx was studied by using ATP-induced $Ca^{++}$-influx, which is mediated by purinergic receptors present in cardiomyocytes (Mubagwa and Flameng, 2002). Both the soluble fraction of PM and $Zn^{++}$ inhibited calcium influx elicited by ATP. However also KCl induced calcium influx known to be receptor-independent and driven by membrane depolarisation and opening of calcium channels was affected by in vitro exposure to PM filtrate. Our data show an effect of the PM-soluble fraction and of $Zn^{++}$ suggesting an effect of soluble metals on calcium channels and ATP receptors-channels. We confirmed earlier observations that soluble metals inhibit the calcium entrance from the extracellular medium by using thapsigargin, an inhibitor of calcium ATPase on the endoplasmic reticulum and Bayk 8443, a dihydropyridine receptor agonist. Both compounds induced a calcium influx from the intracellular storage and extracellular compartment, respectively. PM filtrate and $Zn^{++}$ failed to inhibit intracellular calcium influx elicited by thapsigargin. In contrast, they completely abolished the extracellular calcium influx elicited by the dihydropyridine agonist Bayk 8443.

In summary our data further contribute to the understanding how particulate air pollution causes increased cardiovascular morbidity and mortality in individuals suffering from previous myocardial injury (Peters et al, 2004; Pope et al, 2004). Apart from exerting direct effects on the heart, our studies using a high dose of PM in a susceptible animal model provide evidence that recovery from a myocardial ischemia can be decreased after pre-exposure to PM. The exact mechanism and the nature of the responsible constituent remains an area for further research, but both PM soluble metals and pulmonary inflammation play a role in this process. It remains also open for further study whether this effect also occurs at relevant exposure levels by inhalation.

**ACKNOWLEDGEMENTS**

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Bilthoven. We thank Ton van de Kuil, John F. Boere, Daan Leseman, Jan Bos and Jan Dormansfor their technical assistance in animal studies. We also thank Mrs Konrad and Mrs Nanoaweis for their technical assistance in the calcium measurements. Karim Bagaté was a post-doctoral fellow supported by BWPlus 20018 "Systemic effects *of particles".*

Legends to Tables & Figures

**Figure 1 Recovery of left ventricular developing pressure (LVDP) in isolated rat hearts is impaired in PM and LPS treated rats.** Graphs shows time course of LDVP during and after 35 min occlusion of the coronary artery. Results are given as means ± SEM of (5) individual experiments for each time point. Statistical analysis was done by one-way ANOVA with repeated measures followed by a student Newmann-Keuls test for multiple comparisons. ** and ## $p<0.01$ versus saline.

**Figure 2 Histological appearance of rat myocardium from control (A and B) and occluded heart (C).** Normal histology using H&E **(A)** rendered a green autofluorescence under UV light **(B),** whereas early ischemic changes were identified under fluorescent light microscopy by a typical yellowish auto-florescence **(C).** Ischemic contraction bands were a common feature. No differences in ischemic changes were observed in hearts from control or treated animals. (H&E, 400x).

**Figure 3. Direct effect of infusion of $ZnSO_4$-solution (10 µM) negatively affects LDVP (A) and heart rate (B) in isolated rat hearts.** The Zn-solution was infused into the heart during 10 min to reach a 10 uM concentration in the medium, after which Zn-free medium was supplied during the rest of the perfusion time. No occlusion was applied in this experiment. Results are given as means ± SEM of (5) individual experiments for each point. Statistical analysis was done by one-way ANOVA with repeated measures followed by a student Newmann-Keuls test for multiple comparisons. * $p<0.05$ versus control.

**Figure 4. Receptor-independent (A) and receptor dependent (B) elicited calcium influx in cardiomyocytes.** Cultures of H9C2 cells were treated at 30 min intervals with KCl (50 µM) or ATP (100 µM) and the intracellular Ca-concentration was measured byl recording the fluorescence as the mean of 7-12 individual cells and shown on the left part of the graph; The right parts shows the quantification of results of two subsequent stimulations and are given as means ± SEM of (5-17) individual experiments for each point. Statistical analysis was done by paired student test.

**Figure 5. PM filtrate induces a steady increase of baseline intracellular calcium levels in cardiomyocytes and is inhibited by a Ca-channel blocker.** Cultures of H9C2 cells were treated with PM at different concentrations and showed a slow increase of intracellular calcium. The addition of Nifedipine (2µM) was able to block this baseline increase up to the

highest concentration, showing that Ca-channels are involved in the inward leaching of Ca. Results are given as means ± SEM of (5-9) individual experiments for each point. Statistical analysis was done by one-way ANOVA with repeated measures followed by a student Newmann-Keuls test for multiple comparisons. *** $p<0.001$ versus control.

**Figure 6. PM filtrate and $Zn^{++}$ inhibit KCL and ATP induced Ca-increase in cardiomyocytes.** The left part of the graph shows typical recordings of repetitive KCl or ATP additions to cardiomyocytes before (period 1) and after (period 2) addition of PM filtrate (50-100 µg/ml) or Zn (50 µM). The right part shows quantification of data from multiple experiments and is given as mean ± SEM of (5-15) individual experiments for each point. Statistical analysis was done by one-way ANOVA with repeated measures followed by a student Newmann-Keuls test for multiple comparisons, or paired student test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus control.

**Figure 7. PM inhibits calcium influx induced by Bayk 8344 in cardiomyocytes.** In this experiment BayK (5 µM) was used to stimulate intracellular uptake of Ca and is depicted as control in the left panel. Period 1 and 2 refer to application of thapsigargin in absence and in the presence of PM filtrate (100µg/ml) receptively. Thapsigargin is used to release the intracellular calcium stores. Results are given as means ± SEM of (5-9) individual experiments for each point. Statistical analysis was done by one-way ANOVA with repeated measures followed by a student Newmann-Keuls test for multiple comparisons, or paired student test. * $p<0.05$, ** $p<0.01$ versus control.

**Table 1: Effect of particle and LPS instillation on physiological parameters during the reperfusion period of the isolated perfused heart**

| | | Coronary Flow (ml/mim) | | | Heart rate (bpm ) | | | LVDP (mmHg) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **n** | **saline** | **PM** | **LPS** | **saline** | **PM** | **LPS** | **saline** | **PM** | **LPS** |
| **Baseline** | **5** | 9.8 ± 1.6 | 10.8 ± 2.1 | 10.6 ± 1.5 | 246 ± 15.4 | 260 ± 30.0 | 275 ± 20.0 | 115 ± 7.9 | 78 ± 10.9[a] | 98 ± 7.9 |
| **5 min** | **5** | 7.9 ± 1.0<br>5.8 ± 0.9[b] | 10.2 ± 2.1<br>9.9 ± 2.5 | 7.7 ± 1.55<br>5.6 ± 0.7[b] | 298 ± 34.3<br>304 ± 25.0 | 273 ± 14.0<br>274 ± 20.6 | 251 ± 27.2<br>248 ± 32.6 | 103 ± 11<br>75.6 ± 10.3[b] | 63.3 ± 25[a]<br>57.7 ± 20[a,b] | 73.8 ± 6.6[a]<br>53.2 ± 8.1[a,c] |
| **60 min** | **5** | 4.8 ± 0.6[c] | **11.2 ± 3.7[a]** | **4.5 ± 0.9 [b]** | 242 ± 18.5 | 273 ± 31.9 | 242 ± 10.7 | 72 ± 9.0[b] | 45 ± 13.1[a,b] | 39.4± 6.7[a,c] |
| **120 min** | **5** | | | | | | | | | |

Results are given as means ± SEM of (5) individual experiments for each point. Statistical analysis was done by two way ANOVA with repeated measures followed by a student Newmann-Keuls test for multiple comparisons. [a] $p<0.05$ vs saline, [b] $P<0.05$ and [c] $P<0.01$ versus baseline in the same group

**Fig 1**

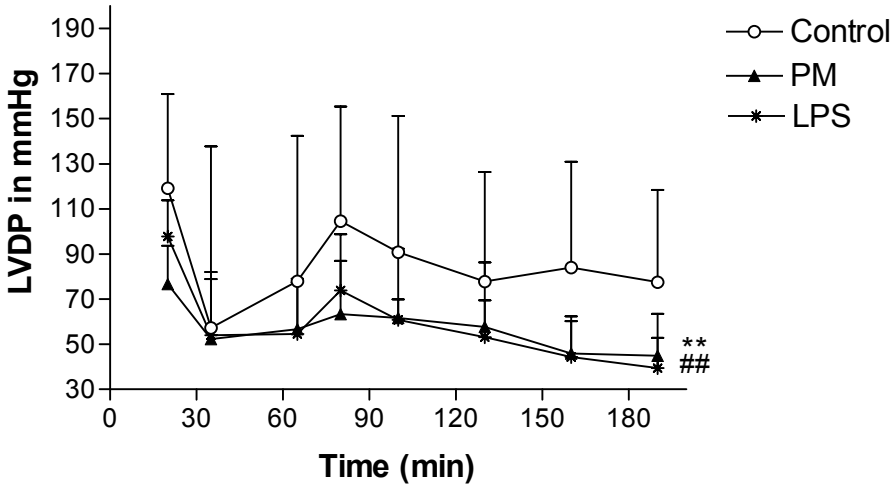

LVDP in mmHg
190
170
150
130
110
90
70
50
30
0
30
60
90
120
150
180
Time (min)
Control
PM
LPS
**
##

**Figure 2.**

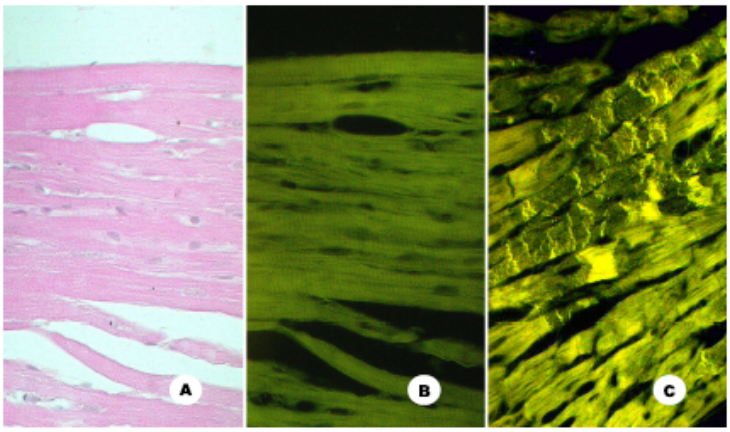


**Fig 3A**

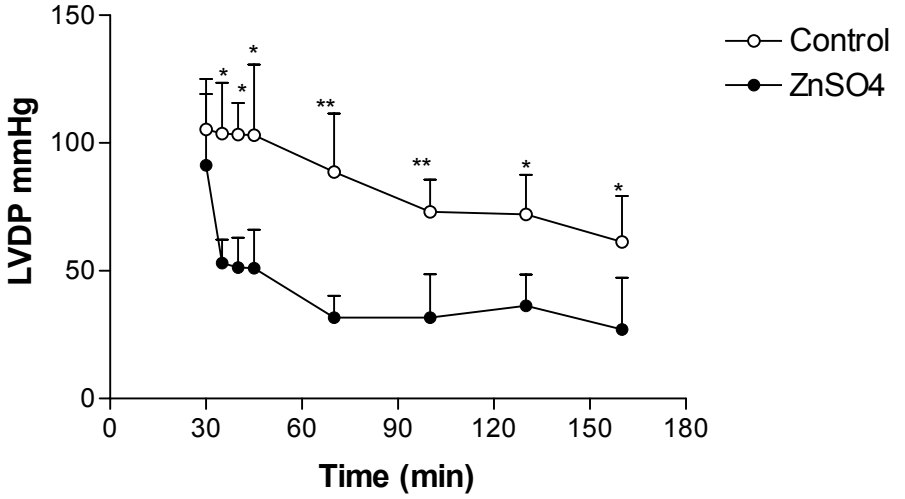

LVDP mmHg
Time (min)
Control
ZnSO4
150
100
50
0
0
30
60
90
120
150
180
*
*
*
**
**
*
*

**Fig 3B**

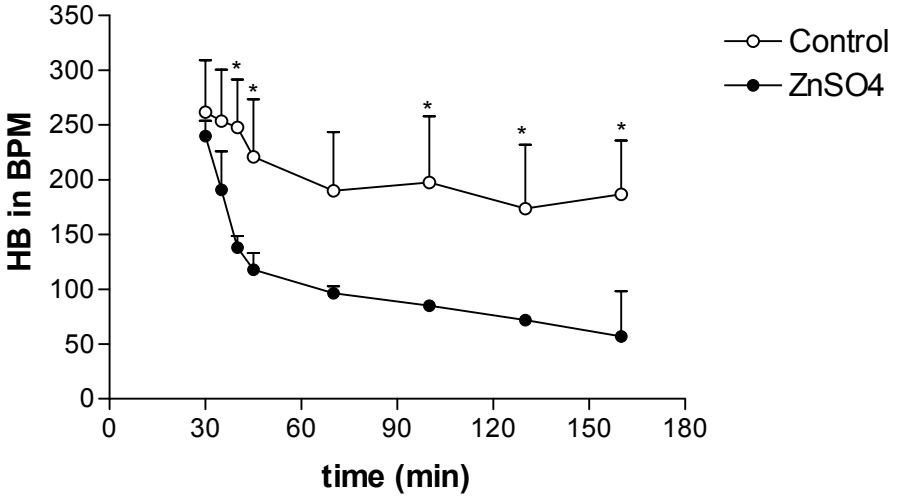
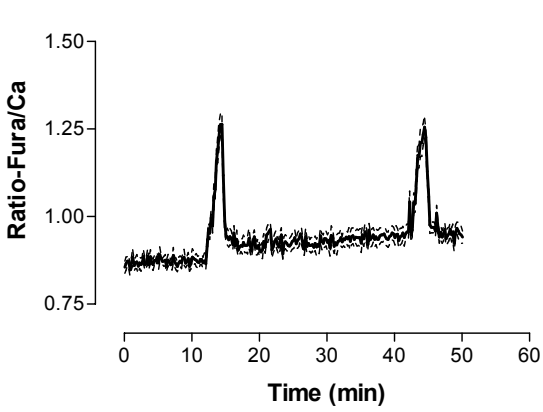

HB in BPM
time (min)
Control
ZnSO4
350
300
250
200
150
100
50
0
0
30
60
90
120
150
180
*
*
*
*
*

**Fig. 4 A**


Ratio-Fura/Ca
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1.25
1.00
0.75
0
10
20
30
40
50
60
Time (min)

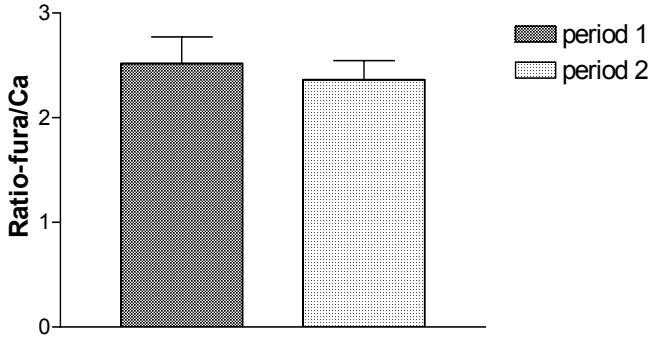
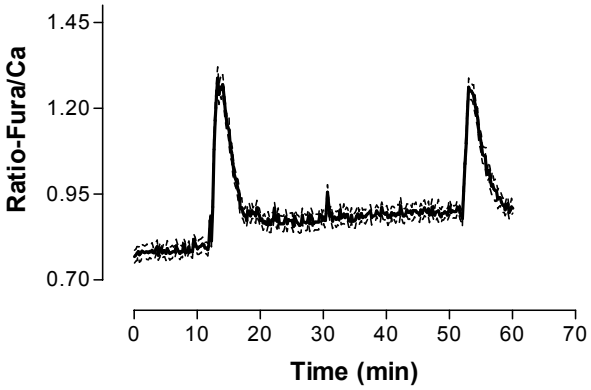

Ratio-fura/Ca
3
2
1
0
period 1
period 2

**Fig. 4 B**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0.95
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0
10
20
30
40
50
60
70
Time (min)

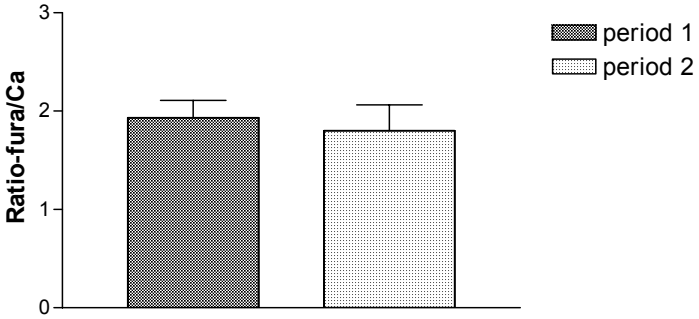

Ratio-fura/Ca
3
2
1
0
period 1
period 2

**Fig.5**

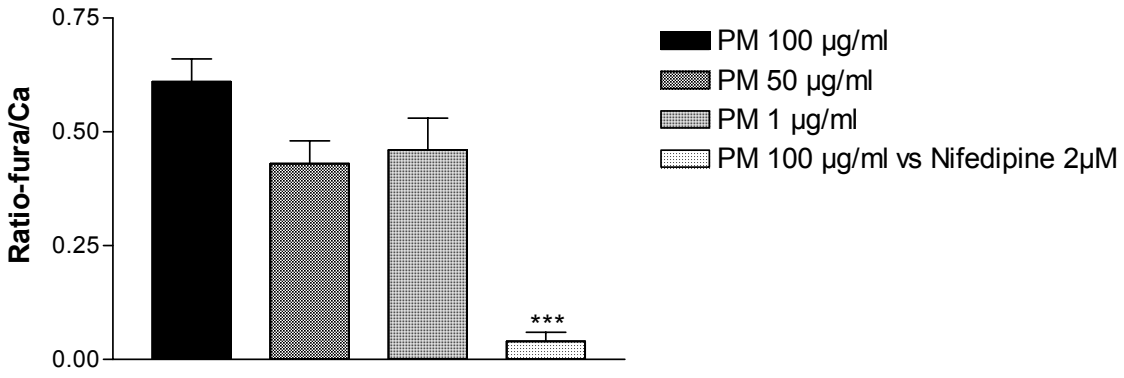

0.75
0.50
0.25
0.00
Ratio-fura/Ca
***
PM 100 µg/ml
PM 50 µg/ml
PM 1 µg/ml
PM 100 µg/ml vs Nifedipine 2µM

**Fig. 6 A**

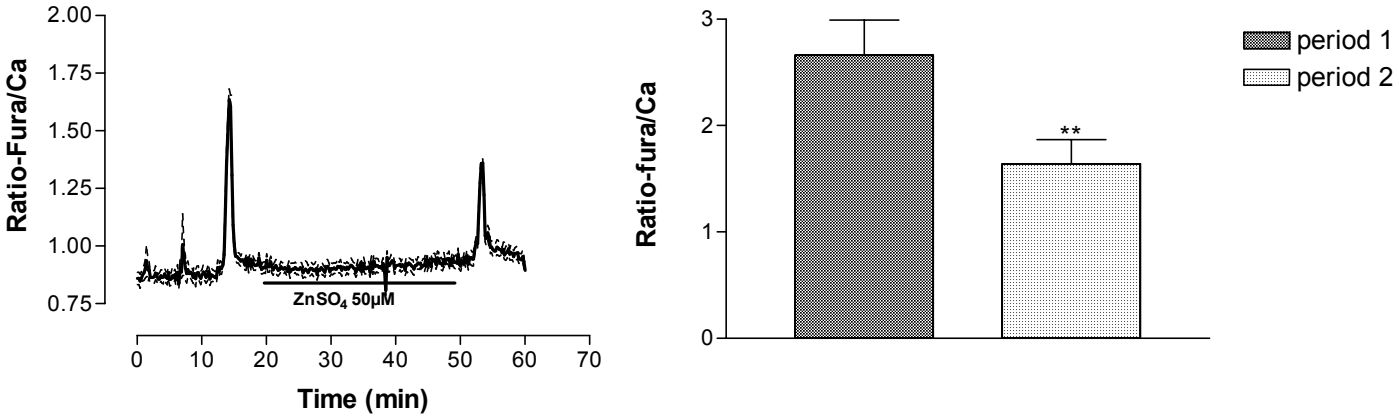

Ratio-Fura/Ca
2.00
1.75
1.50
1.25
1.00
0.75
ZnSO4 50µM
0
10
20
30
40
50
60
70
Time (min)
Ratio-fura/Ca
3
2
1
0
**
period 1
period 2

**Fig. 6 B**

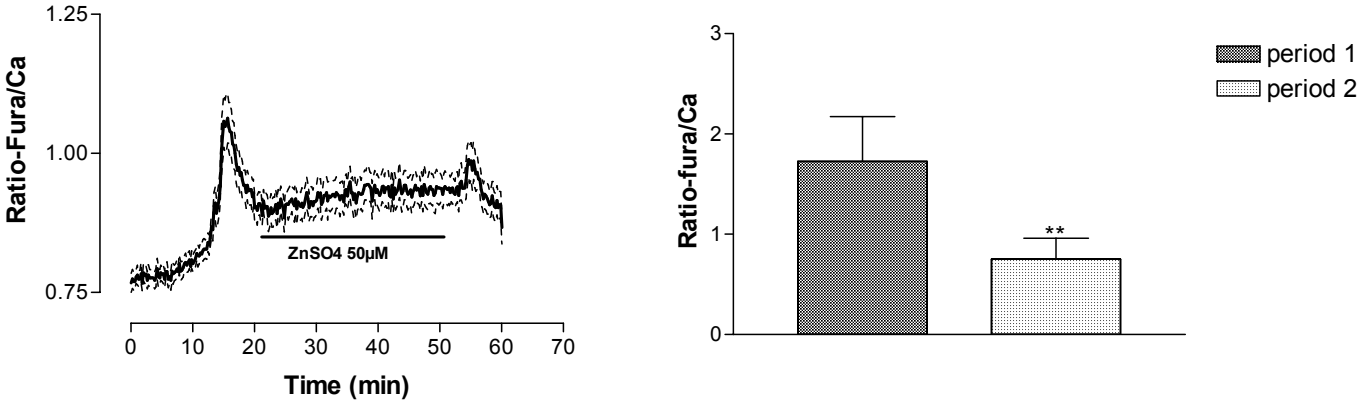

Ratio-Fura/Ca
1.25
1.00
0.75
ZnSO4 50µM
0
10
20
30
40
50
60
70
Time (min)
Ratio-fura/Ca
3
2
1
0
**
period 1
period 2

**Fig. 6 C**

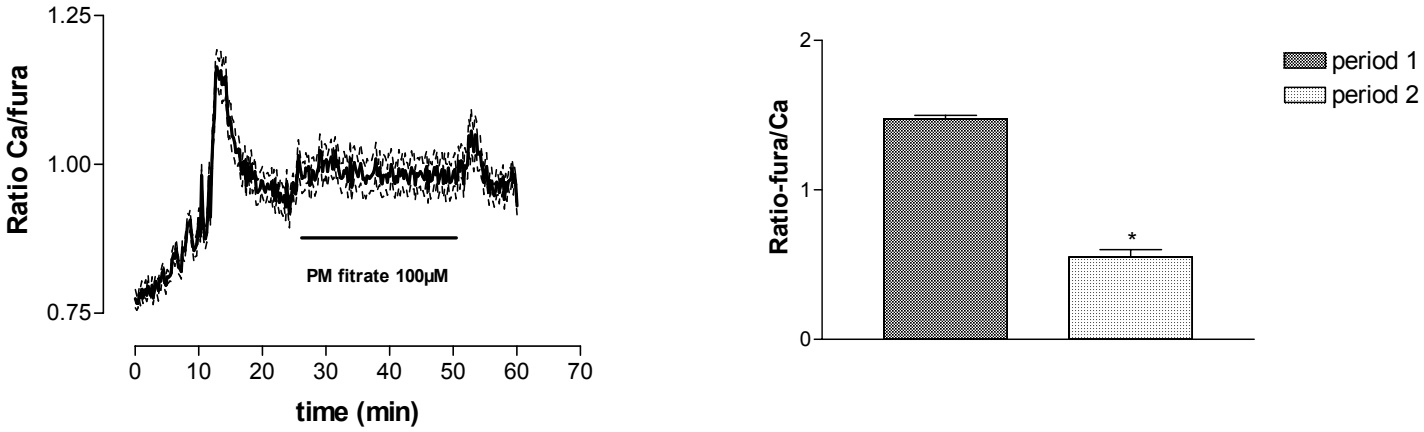

Ratio Ca/fura
1.25
1.00
0.75
PM fitrate 100µM
0
10
20
30
40
50
60
70
time (min)
Ratio-fura/Ca
2
1
0
*
period 1
period 2

**Fig 6 D**

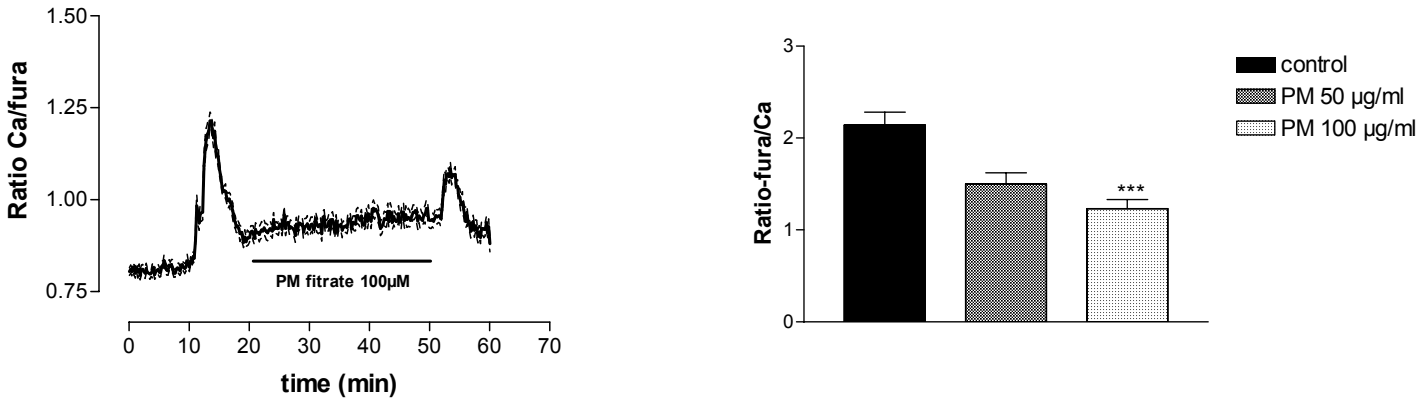
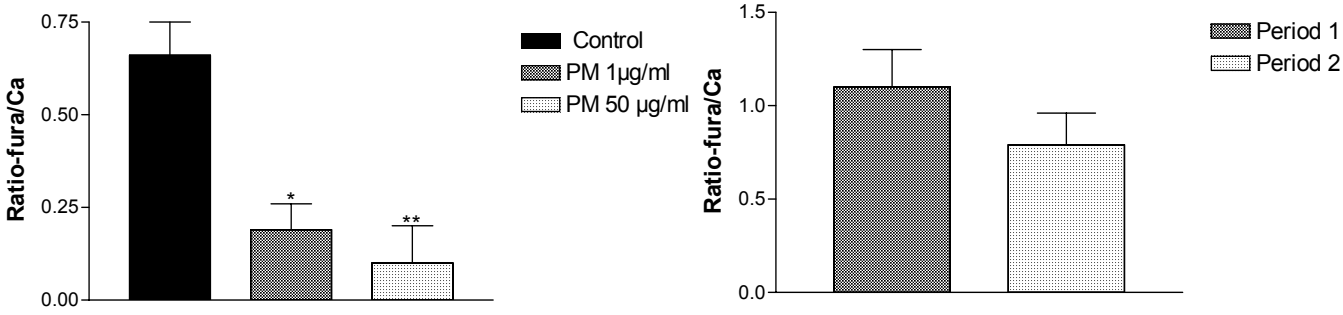

Ratio Ca/fura
1.50
1.25
1.00
0.75
PM fitrate 100µM
0
10
20
30
40
50
60
70
time (min)
Ratio-fura/Ca
3
2
1
0
***
control
PM 50 µg/ml
PM 100 µg/ml

**Fig 7**


Ratio-fura/Ca
0.75
0.50
0.25
0.00
*
**
Control
PM 1µg/ml
PM 50 µg/ml
Ratio-fura/Ca
1.5
1.0
0.5
0.0
Period 1
Period 2

# Chapter 5

## General Discussion

Ambient particulate matter exposure has been implicated in epidemiological studies to be responsible for adverse health effects (Daniels et al, 2000; Pope et al, 2002). These studies estimated that an increase of 10 μg/$m^3$ $PM_{2.5}$ resulted in mortality increase of 1.4 % while respiratory diseases such as bronchitis or asthma exacerbation increase by much as 4 % (WHO, 1999). Various hypotheses exist but no clear biological mechanism or pathway is at hand to explain the can produce these health effects observed in epidemiological studies. Toxicological and epidemiological evidence suggest that fine ($PM_{2.5}$) and ultrafine ($PM_{0.1}$) fraction is responsible for these adverse effects there seems to be no agreement (Oberdorster et al, 1994; Wichmann et al, 2000). Due to different endpoints in the wide number of studies, the suggestions are that more than one component may be driving the health effects (Donaldson et al, 1998; Dreher, 2000). Recent studies from our laboratory showed the autonomic effects of ambient particles on epinephrine-contracted aorta-rings comparable to carbachol, and that this effect is only partly mediated through endothelial mediators such as as nitric oxide (Knaapen et al, 2000). Also from our group it was shown that ultrafine (20nm) but not fine (120 nm) carbon black particles are able to interfere with $Ca^{2+}$ transport across the cell membrane (Stone et al, 2000). Taken together, this data shows that if particles or particles extracts are able to cross the epithelial barrier in the lung, and reach the heart and aorta they should be able to induce direct effects. Accompanying these directs effects , other such as Seaton et al, (1995) have suggested that inflammation can cause disturbance in systemic blood coagulation and viscosity or can destabilise and cause rupture of atheromatous plaques (Donaldson et al, 2005). The purpose of this study was to test if the previous autonomic effects can occur in a system that preserves the structural integrity of the lung and allows to study particle uptake as well as particle induced release of inflammatory and vaso-active substances. The **isolated perfused rat lung (IPRL)** is suitable for this goal, since this system allows us to i) exclude systemic anti-oxidant back-up from the perfusing blood, allows to study inflammation using PMN migration into tissue and alveolar space, iii) retains structural intergrity necessary to study the effect of ultrafine particles and iv) to sample perfusate that can be investigated for direct biological activity and the presence of particles. Experiments were designed to elucidate inflammation as modulator of lung permeability, translocation of particles and as a generator of vasoactive substances (all induced by instillation by particles.

These studies objectives were to see the effects of particles on the lung and its indirect systemic effects through activation and release of vaso-active substances; and the direct effect of particles by translocation in the lung independent of activation of inflammatory pathways. Particle translocation was monitored by radioactivity of the particles and not by any attached radioactive label. No translocation of Ir particles (17-20 nm) was detected in normal *negative pressure* perfused lungs. However lungs pre-treated with histamine on the endothelial side (1µM ) or $H_2O_2$ (0.5 mM) in the alveolar lumen showed small amounts of radioactivity in the single pass perfusate after a significant lag-time. This particle translocation coincided with increased permeability for DTPA in the histamine perfused and $H_2O_2$ treated lungs.
No translocation of Ir particles (17-20 nm) was detected in normal *positive pressure* perfused lungs. Even lungs pre-treated with histamine on the endothelial side (1µM ) or $H_2O_2$ (0.5 mM) in the alveolar lumen showed no amounts of radioactivity in the recirculating perfusate after a significant lag-time. Addition of PMN in perfusate with PMN activators did not reveal any Ir particles in the perfusate. However we establish an *ex-vivo* inflammatory lung model that can be used in variety of pulmonary investigations. Additionally we could successfully label human PMN's to be used in an isolated perfused rat lung model.
Viewing the effect that ambient particles have on SHR, our data showed that pre-treatment of SHR rats with ambient particles and LPS does affect the ability of the isolated heart to recover after an ischaemic insult caused by coronary occlusion, although no difference was observed. We conclude that effects may be caused by soluble components in the PM preparation. This is supported by our cellular studiesin cardiomyocytes where we observed an effect of PM and metals on intracellular calcium channels.

As mentioned, PM consists of organic fraction, inorganic and metals as well as a biogenic fraction composed by bacterial (Becker et al, 2005). Metals found in ambient particle matter are Fe, Cu, V, Ni, Cr, Al as well elemental carbon along with sulfate and nitrate. It is still unclear which of these constituents are involved in the cardiovascular effects seen in epidemiological studies. PM is able to generate reactive oxygen species due to its constituents (Knaapen et al, 2002). We hypothesise that both direct and indirect effects might contribute to the adverse health effects observed in epidemiological studies. This thesis investigated the hypothesis by using an ex-vivo isolated lung perfusion system, an in vivo model by instilling ambient PM intra-tracheal into SHR as well as looking at cellular model reaction to intracellular $Ca^{++}$ homeostasis after particle treatment. Using these different experimental models help us to elucidate the possible biological mechanisms responsible in particle mediated effects in the human body. The isolated lung perfusion model (negative/positve pressure) allowed us for evaluating if particles are able to translocate

across the alveoli/epithelial layer into the systemic system. The model might be questioned due to artificial perfusion and ventilation of the lung. The lung is also deprived of its autonomic nervous function. Nevertheless, the isolated perfused rat lung model is extensively used to elucidate and investigate basic pulmonary questions. We used this model for detection of inhaled model particles after instillation or inhalation and the behaviour and effects of these particles. There seems to be much speculation about the concept of particle translocation due to the lung defence mechanism and the tight epithelia/endothelial barriers. We accomplished the tracking of the model particles, by using radio-active ultrafine Iridium particles. We initially started our studies with fluorescence latex particles (Data not shown). However, translocation was very small. Ambient particle inhalation is a continuously action (chronicle). Particle translocation might be small, but we argue that chronicle minute translocation of ambient particles into the systemic system might possible result in adverse vascular effects. Previous studies have shown the translocation of nanoparticles from lung to the circulation (Kreyling et al, 2002; Nemmar et al, 2001; Oberdorster et al, 2002), however little consistency is present between human and animal studies (Mills et al, 2005, AJRCCM). So far studies have indicated that both heart and vasculature are affected and it is assumed that systemic oxidative stress plays a pivotal role. This assumption is more based on parallel observations in related fields such as angiogenesis, than on real evidence for systemic oxidative stress. Effects on the vasculature are rapid and may be mediated by release of second messengers from the lung.

Our lung model opened up the possibility to investigate the influx of neutrophils into the lung. Various studies observed the influx of neutrophils in the lavage of animal lungs after instillation of particles. We assumed then that neutrophils might be instrumental in possible particle translocation into the systemic circulation. Lung inflammation might play a key role in lung permeability as been shown by Seeger and colleagues (1995). Neutrophils were isolated from fresh human blood and stable labelled with dye for detecting purpose. This allowed us to monitor the neutrophils' activity in the lung. We however didn't observe any translocation in the positive pressure neutrophil perfused lung model. The positive pressure model might not the ideal model for particle translocation studies. Mediator release from both positive and negative pressure perfusion didn't reveal any significant differences between the experimental and control groups. We postulated earlier that mediator release might also be fundamental in the adverse health effects observed of particles. The perfusion system, especially using plasma expanders and bovine serum albumin might have affected mediator release. The use of ex vivo organs might also produce too little mediators that might not be able to measure. Also the perfusion medium allows only for a short experimental (2 hours) condition. Other mediators which were not use in our study scope might also be of importance in future experiments. The isolated perfused lung could address other functional

parameters regarding particle pollution in future studies. We should note that we used an acute particle inhalation study to assess particle translocation. The real physiological set-up in humans involved constant chronicle exposure to ambient particulate matter that might have far different pathophysiological outcomes than our present study. However our model somewhat reveal possible mechanistic behaviour of particles.

Assessing particle effects further on the isolated heart and vessels, we could clearly see particle or particles constituents having effect on the myocardial contraction or $Ca^{++}$ fluxes. The effects seen with the isolated heart and with the H9C2 cells confirm the speculation that translocated particles or particles soluble fraction can have secondary effects. As the heart is the first organ encounter after blood returns to the circulation, this ex vivo experimental model further confirms a possible mechanism whereby ambient particulate matter can exert biological effects. Our various experimental showed that particle matter can have an effect via particle translocation resulting in direct effects on vascular and heart or that particulate matter constituents having direct effects on the vascular or particulate constituents disturbing calcium homeostasis in cardiac cells or vascular. We hypothesised that the effects of particulate matter may be facilitated by pulmonary inflammation (**Fig 1)**. This was supported by histamine perfusion in the isolated perfused lung model, but on the other hand our model with neutrophils in the perfused lung did not reveal enhanced translocation. Inflammation as induced by soluble LPS had a similar effect on recovery of the heart after ischemia compared to animals that were pretreated with PM (**Chapter 4**). Inflammation might result in an increased epithelium permeability resulting in the crossing of insoluble/soluble ambient particle matter or inflammatory mediators into the systemic circulation.

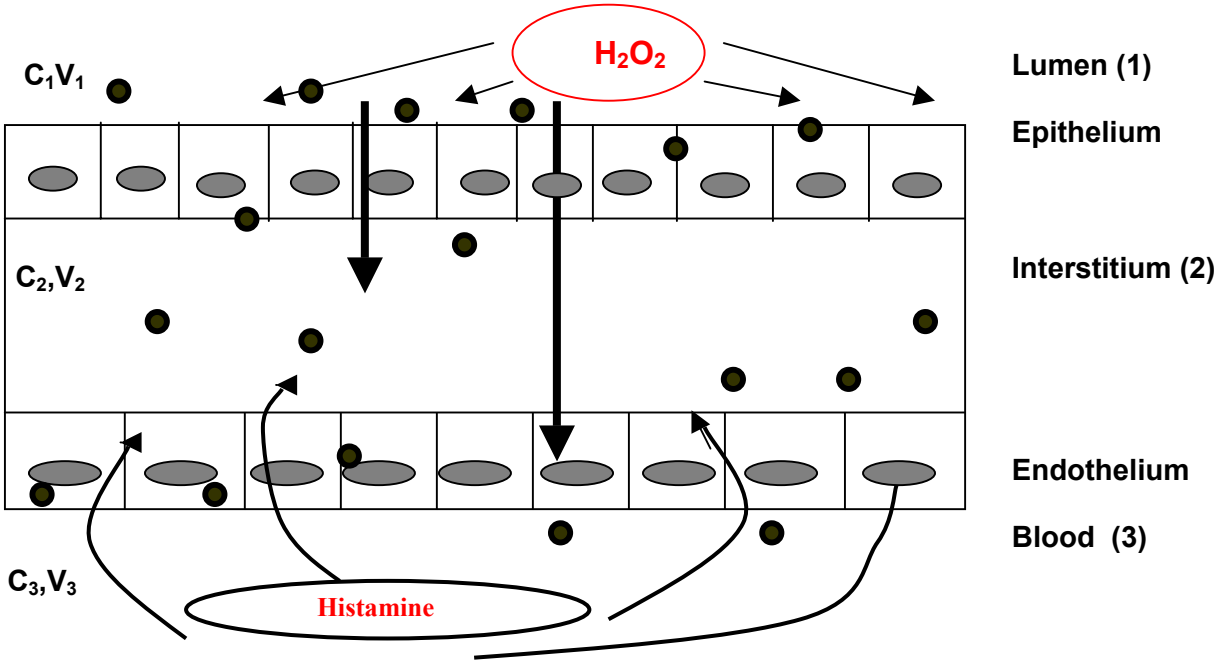


**Fig. 1**

*Illustration of the suggested effects of histamine and hydrogen peroxide on the permeability of the alveoli/epithelium/endothelium barrier.*

Our experimental models are however not without shortcomings and this limits our conclusions. Autonomic nervous control is not playing a role in the isolated heart and lung (so feeback mechanisms through vagal afferent nerves as recently demonstrated by Duffin et al, (2006) are excluded. Furthermore, we made use of artificial perfusion media which cause our experimental models to last only for a limited period of 2-3 hours. In some cases this might be too short for certain biological effects such as inflammation. The cell line H9C2 is cellular model represent a transformed cell line which is not fully identical to the cardiovascular cell population. Irrespective of the above limitations of the models in this study, we have demonstrated that particulate matter might have both indirect and direct effects that are consistent with the adverse effects observed in epidemiological studies. The study established both in ex vivo and in vivo models that particle and constituents can exert biological effects. The biological mechanism of particles on the cardiovascular system might be initiated through various pathways which include particle translocations, particle induced inflammation that effect cardiac parameters, particle constituents affecting vascular function as well as inducing intracellular calcium fluxes. More research however is needed to further elucidate the biological mechanism and mediators that cause the effects of ambient particles on the cardiovascular system.

**Table 1. Overview of current data on particle translocation from the respiratory system to the circulation.**

| Particle size | Particle type | T | Details | Reference |
|---|---|---|---|---|
| 18 nm | Iridium | -/+ | < 0.2 % within 24 hrs after cessation of inh exposure ( ) | Kreyling 2002 |
| 8 nm | Gold | + | Gold particles were found in plasma space after 15 min injection into the heart | Konig et al , 1994 |
| 18 nm | Iridium | -/+ | Up to 3 % in IPL after aerosol exposure for 1 hr | Meiring et al, 2005 |
| 35 nm | Carbon | + | Significantly amount of $^{13}C$ UFP | Oberdorster et |

| | | | | |
|---|---|---|---|---|
| | | | accumulated in liver after 0,5 hours after inhalation | al, 2002 |
| 5-20 nm | Gold, coated with Albumin | -/+ | Intravenously administered, increased translocation after LPS administration (possible receptor mediated transcytosis) | Heckel et al, 2004 |
| 30 nm | Gold | + | Large amounts particles found in pulmonary capillaries after inhalation | Berry et al, 1977 |
| 80 nm | Albumin Nanocoillod | +/- | Less than 3 % of intratracheal instilled dose found in blood | Nemmar et al, 2001 |
| 100 nm | Labeled nano carbon | - | No translocation. Most of instilled dose stayed in lung | Mills et al, 2005 |
| 24, 110 , 190 nm | Carboxylated and amine fluorescent | +/- | Intratracheal instillation no translocation, histamine administration result in translocation from capillary to alveoli | Nemmar et al, 2005 |

Further research is needed to understand the processes and particle properties that determine translocation. Particles might translocate across the epithelial-endothelial barrier via various transport modes. Possible routes are via passive paracellular migration, vesicle traffic, endocytosis or transcytosis (Gumbleton, 2001). The review by Oberdorster et al, (2005) discuss the possible mode of translocation by various studies (**Table 1**). Particles coated by albumin might translocate via the caveolae in a receptor mediated transcytosis. Albumin and phospholipids are believed to facilitate some particles translocation. A number of different endocytic pathways that might facilitate particle translocation are discussed by Rejman et al, (2004). He proposed that latex microspheres < 200 nm might be iunternalize by clathrin coated pits and that bigger microspheres might translocate via caveolae's. Caveolae are abundant in lung capillaries and alveolar type I cells. In a paper by Kato et al, 2003, polystyrene latex beads were coated with lecithin and intratracheal administered to rats. They observed that both lecithin coated and uncoated latex beads (240 nm) were incorporated into alveolar macrophages and that lecithin coated beads incorporated as well into the Type I and II alveolar epithelial cells. This transfer of the latex beads are suppose to be mediated by transcytosis.

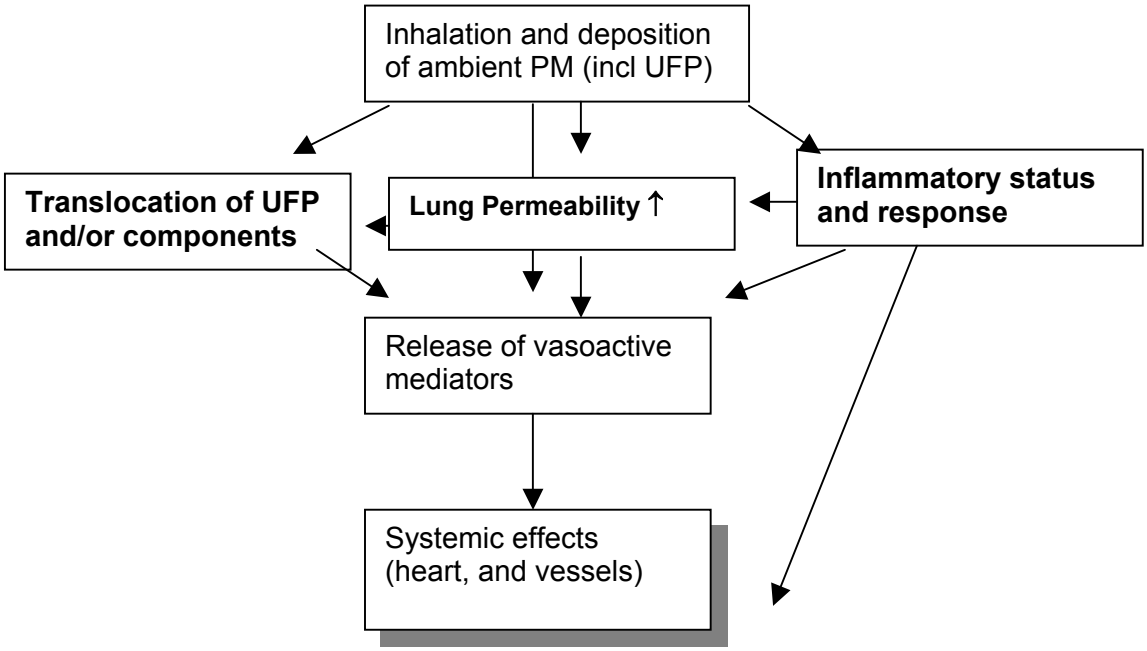
Comparing our data with other in vitro or in vivo data (Table **1** ), shows that our translocation model somehow conforms to similar study by Seeger and colleagues (1995). The latter study and ours clearly observed that hydrogen peroxide increase lung endothelial and epithelial permeability. We suggest that this facilititates the transport of nanosized particles to cross from the alveoli into the systemic circulation. In a similar type of study Heckel et al, (2004) showed that inducing an acute type of lung injury by infusion of LPS can change the permeability of the lung and other organs. They observed that 4 nm gold particles cross the alveoli barrier even from the systemic circulation directly into the alveoli. Although this is not same direction we postulating, the study does support our hypothesis that inflammation will mediate organ permeability and translocation. Also Nemmar et al, (2005), showed increased particle translocation in the rabbit lung when perfused with histamine, showing that particles can translocate from the perfusate into the alveoli.

Our experimental work may be connected to real life situation, since patients with COPD and systemic inflammation (e.g. diabetes) are at risk for particle effects after inhalation. In a recent paper of Frampton and colleagues (2006), it was shown that ultrafine particles result in expression of leukocyte adhesion molecules. This would further aggravate systemic inflammatory conditions in susceptible individuals.

To further advance the understanding of particle translocation and cardiovascular effects it would be interesting to use the **isolated rat lung-heart** preparation. Valuable data could be extracted from this model since it can demonstrate at the same time the direct effects of particles on the myocardium and effects of mediators on the myocardium. As mentioned already, the advantage of using isolated perfused organs, is that we excluding hormonal and neural influence on the perfused organ. The isolated lung-heart model could even corobarate the actual effect of model and ambient particles on the circulatory system. The model allows to monitor perfusate from lung and there subsequent effect on the heart. This would probable answer the question whether direct or indirect pathways cause the biological effects. In vivo models should address more chronic inhalation studies as acute studies are not fully conclusive. More advance experimental conditions should be initiated to elucidate particle interactions with other organs that play a role in hemostasis such as the liver and kidneys.

**General conclusion**

Although these data do not allow definitive conclusions on the exact mechanism and the importance of systemic translocation of UFP as a mechanism in adverse effects of PM, we do confirm that ultrafine particles can translocate from the lung into the circulation using the isolated perfused rat lung upon pharmacological mediation. Permeability of the lung barrier to ultrafine particles seems to be controlled both at the epithelial and endothelial level and conditions that affect this barrier function such as inflammation may affect translocation of UFP. The conditions under which this occur mimics conditions that are met in diseased, susceptible subjects including asthmatics and COPD-patients. However apart from translocated nanoparticles to the bloodstream, several other mechanisms need to be considered in causing systemic effects. Infact in this study pre-treatment of normal and spontaneous hypertensive rats, showed that both inflammatory mediators (released from the lung) and soluble particle components (metals) are able to indirectly affect cardiac and vascular function, by various mechanism. The relative importance of these mechanisms is highly dependent on the pro-inflammatory condition of the subject, which determines the cardiovascular responsiveness as well as the lung permeability. The latter is a crucial process in the translocation of UFP and probably also for particle components such as transition metals.



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