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# NITRIC OXIDE IN EXPERIMENTAL PULMONARY EMBOLISM

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## TO MY FAMILY

"Not everything that counts can be counted,  
and not everything that can be counted counts."

(Sign hanging in Albert Einstein's office at Princeton)

## **ABSTRACT**

Nitric oxide (NO) is an important modulator of the pulmonary circulation both at basal state and in pulmonary hypertension. Low levels of NO are detectable in exhaled gas which is believed to mirror pulmonary NO formation and elimination. Pulmonary embolism is a disease characterised by pulmonary hypertension, and thereby increased afterload of the right ventricle, and by disturbed gas exchange which produces hypoxemia. The role of NO in acute pulmonary embolism was studied in two animal models.

The fraction of NO in exhaled gas increased dramatically after induction of acute pulmonary embolisation with both gas and solid emboli. It was found that approximately 50% of the increased exhaled NO could be reversed by normalising the airway/alveolar carbon dioxide concentration, thus indicating a regulatory role of carbon dioxide on pulmonary NO production in this condition.

Endogenous NO production exerts a protective effect in acute pulmonary embolism since it was found that inhibition of endogenous NO production in combination with pulmonary embolisation resulted in a severely augmented hemodynamic response and significantly impaired the survival in this condition. Therefore it was further hypothesised that exogenous NO might be protective in this condition.

NO donor compounds, some of which were novel organic nitrites, with increased selectivity towards the pulmonary circulation were developed. Intravenously administered organic nitrites reduced the pulmonary hypertension and relieved the strain on the right ventricle in acute pulmonary embolism without adverse effects in the form of systemic hypotension, methaemoglobin formation and tolerance development. Methods for identification and characterisation of organic nitrites were described, including a novel HPLC-NO/nitrite analysis.

These studies show that exhaled NO is increased after acute pulmonary embolism thus emerging as a potential diagnostic aid in this condition. Endogenous NO is protective in acute pulmonary embolism which provides further knowledge on the role of NO in the pulmonary circulation. Exogenous NO, in the form of certain organic nitrites, exerts beneficial effects in acute pulmonary embolism, thus rendering organic nitrites as a potential future life-saving treatment in acute pulmonary embolism. Future studies will investigate the effects of organic nitrites in experimental models of other life-threatening diseases with compromised pulmonary circulation.

## LIST OF PUBLICATIONS

The thesis is based on the following studies, which are referred to in the text by their Roman numerals:

- I Agvald P, Adding LC, **Nilsson KF**, Gustafsson LE, Linnarsson D (2006). Increased expired NO and roles of CO<sub>2</sub> and endogenous NO after venous gas embolism in rabbits. *Eur J Appl Physiol.* 97:210-5.
- II **Nilsson KF**, Gustafsson LE, Adding LC, Linnarsson D, Agvald P (2007). Increase in exhaled nitric oxide and protective role of the nitric oxide system in experimental pulmonary embolism. *Br J Pharmacol.* 150:494-501.
- III **Nilsson KF**, Lundgren M, Agvald P, Adding LC, Linnarsson D, Gustafsson LE. Formation of new bioactive organic nitrites and their identification with gas chromatography-mass spectrometry and liquid chromatography coupled to nitrite reduction. *Manuscript.*
- IV **Nilsson KF**, Gustafsson LE. Treatment with new organic nitrites in pulmonary hypertension of acute experimental pulmonary embolism. *Manuscript.*

## LIST OF ABBREVIATIONS

FENO	Fraction of nitric oxide in mixed exhaled gas
GC-MS	Gas chromatography-mass spectrometry
HPLC	High performance liquid chromatography
L-NAME	<i>N</i> <sup>G</sup> -nitro-L-arginine methyl ester
LC-NO	High performance liquid chromatography coupled to on-line nitrite reduction
MAP	Mean systemic arterial blood pressure
NO	Nitric oxide
NOS	Nitric oxide synthase
PDNO	Propanediol nitrites solution
PEEP	Positive end-expiratory pressure
V/Q	Ventilation/perfusion

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## **1. INTRODUCTION**

The primary function of the lung is gas exchange, i.e. saturating the haemoglobin in the blood with oxygen and excreting carbon dioxide produced by the cells of the body. Normally the pulmonary circulation is a high flow-low pressure system and the right ventricle is therefore adapted to pumping large volumes of blood against a low afterload (131). Normally the perfusion is matched to the ventilation, thus optimising gas exchange (131). The most important mechanism for ventilation-perfusion matching is hypoxic vasoconstriction, first described by von Euler & Liljestrand (240). Another important regulator of the pulmonary circulation is the endogenous pulmonary vasodilator nitric oxide (NO) which has been suggested to take part in the matching of ventilation and perfusion (6, 177). The present thesis investigates pulmonary embolism where the resistance in the pulmonary circulation is abnormally increased, thus challenging the right ventricle, and where the gas exchange is severely disturbed due to a mismatch between ventilation and perfusion resulting in hypoxemia. Especially the studies concerned the role of NO in experimental pulmonary embolism.

### **1.1. NITRIC OXIDE**

With the discovery of endothelial-derived relaxing factor and its elucidation as biologically formed NO gas, two new principles were established: signalling via gas molecules and the role of the endothelium in vascular regulation (76, 101, 169). Since then a multitude of papers has been published on the role of NO in biological systems and the reader is referred to them. Importantly, the role of NO in vascular regulation in health and disease (148), its roles in the nervous system (116, 246), its diverse function in intracellular signalling and the immune response (77), the biological chemistry of NO (93) and biochemistry of NO (211, 212) have been reviewed elsewhere. In addition to NO formation by NO synthases (NOS), NO can also be produced independently of NOS from inorganic nitrite and nitrate in mammals (132). Phosphodiesterases, and specifically phosphodiesterase 5, play an important role in the biological functions of NO (73).

#### **1.1.1. Nitric oxide in the lung**

A fairly recent review deals extensively with the role of NO in the pulmonary system, including exhaled NO (189). All three isoforms of NOS are expressed in the lung (78). Endothelial NOS is located to the vascular endothelium, thus regulating vessel diameter (196), but found also in type II alveolar epithelial cells (172) and bronchial epithelial cells (197). Neuronal NOS is found in neurons within the lung (117), where it mediates nerve-induced smooth muscle relaxation (24). Pulmonary macrophages contain inducible NOS which produces NO in cytotoxic amounts, thereby involved in host defence (107) but inducible NOS can be expressed in several other non-immune type cells in the lung upon stimulation (19, 189). Several phosphodiesterases including phosphodiesterase 5 have been identified in human pulmonary artery and human lung (55, 186). The phosphodiesterase 5 is abundant in the lung which therefore makes treatment of some pulmonary diseases, e.g. primary pulmonary hypertension, with phosphodiesterase 5 inhibitors logical (2, 44, 182).

*Exhaled NO*

The discovery in 1991 that NO can be measured in low levels in exhaled gas in humans and several animals (88) started a new research field. The NO concentration in exhaled breath is a result of the pulmonary NO production since NO in exhaled breath can still be measured after stopping the circulation (88). Exhaled NO derives from enzymatically produced NO since it is abolished by NOS inhibition (88). Therefore the level of exhaled NO can be seen as an on-line measurement of pulmonary NO production and elimination. Nowadays exhaled NO can be measured in humans with a handheld, small apparatus rendering exhaled NO as an easy, non-invasive and fast bed-side examination (92). Measurement of exhaled NO has been very valuable in experimental studies including investigations of the regulation of pulmonary NO production by oxygen, carbon dioxide and pulmonary stretching (85, 210). Exhaled NO has also been used for monitoring of NO formation from NO donors such as organic nitrates and organic nitrites (35, 98, 135, 173). In healthy humans, single-breath measurements of exhaled NO indicated that NO in exhaled breath arises from terminal and respiratory bronchioles (179). In the rabbit, a major part of exhaled NO originates from calcium-dependent NO synthases and in particular endothelial NOS (178, 233). It was early shown that exhaled NO is increased in humans suffering from inflammation in the airways, e.g. asthma (16, 113, 175, 180). In asthmatics the elevated exhaled NO is mainly derived from the lower airways (111). The increase in exhaled NO in asthmatics and active pulmonary sarcoidosis have been attributed to induction of inducible NOS since the expression of this enzyme is induced in inflammation by inflammatory mediators and cytokines and inhibited by glucocorticoid treatment (114, 153, 244). Other diseases that are associated with increased exhaled NO include respiratory viral infections (20), pneumonia in ventilated patients (7), bronchiectasis (112) and active fibrosing alveolitis (170, 189). Chronic heart failure with pulmonary abnormalities (3, 9), pulmonary hypertension, cystic fibrosis and ciliary dyskinesia are associated with low exhaled NO levels (21). The exhaled NO levels in chronic obstructive pulmonary disease are high or normal and high in exacerbations (10), however concurrent smoking is interfering by decreasing exhaled NO values (21, 180). Endogenous NO may not be important in regulating the tonus of the airways in basal state, but endogenous NO is counteracting bronchoconstrictor stimuli, e.g. histamine and ovalbumin (163, 174). Inhaled NO counteracts pharmacologically induced bronchoconstriction (66). Since exhaled NO is increased immediately after allergen-challenge and in asthmatics (175, 180) a protective role of endogenous NO has been suggested but damaging effects by for example peroxynitrite formation cannot be excluded (213). Since pulmonary NO production, measured as exhaled NO, is changed in several diseases affecting the lung and is regulated by airway/alveolar gases the response in exhaled NO in acute pulmonary embolism was studied in the present thesis work.

*NO in the pulmonary circulation and in ventilation/perfusion matching*

Endogenous NO continuously regulates the pulmonary and systemic circulations in several species including humans and rabbits, as evidenced by NOS inhibition in these species which increases pulmonary and systemic vascular resistances (177, 206). Furthermore endogenous NO is a modulator of the hypoxic vasoconstriction and

pulmonary hypertension induced by a thromboxane A<sub>2</sub> analogue, U46619 (177, 228). Local regulation of pulmonary blood flow is attenuated by NOS inhibition (190). Pulmonary NO production has been shown to be regulated by airway CO<sub>2</sub> and O<sub>2</sub> (6). Hypoxia (<10%) and hypercapnia, indicating reduced ventilation/perfusion (V/Q) ratio, have been shown to decrease pulmonary NO production (88, 210). Normoxia and hypocapnia, indicating increased V/Q ratio, have been shown to increase pulmonary NO production (243). The phenomenon of reciprocal changes in expired CO<sub>2</sub> and NO has been shown both for hypocapnia and hypercapnia *in vivo* and in isolated lungs (29, 33, 243), however the mechanism still remains to be elucidated. It has been shown both *in vivo* in humans and animals and in isolated lungs that severe hypoxia (<10%) reduces exhaled NO (11, 85, 88, 100, 194). This effect is probably derived from that the enzymatic formation of NO involves molecular oxygen (67). Interestingly, hypoxia in combination with hypercapnia decreases exhaled NO even further (243). In addition, pulmonary stretch by increasing the functional residual capacity increases pulmonary NO production (210) and inhibition of stretch-dependent calcium channels by gadolinium chloride decreases pulmonary NO production and induces pulmonary hypertension (5). Therefore, it has been suggested that endogenous NO is an important modulator of V/Q matching (6, 177, 190), but this has been challenged since inhalation of a NOS inhibitor decreased the ventilation-perfusion heterogeneity in healthy dogs (29). One explanation to this discrepancy may be that endogenous NO does not regulate basal pulmonary vascular resistance in dogs (192), in contrary to in humans and rabbits (177, 206). Nevertheless, these studies show that endogenous NO production is an important pulmonary vasodilator both in health and in pulmonary hypertension; therefore it would be important to study its role in acute pulmonary embolism.

### **1.1.2. Nitric Oxide Donors**

Nitric oxide donors are compounds that can release NO to the body and may be used as treatments in condition where additional NO is thought to be beneficial. Inhaled NO gas is special in that it is a way to deliver the actual NO molecules specifically to the lung and the blood that passes the lung. There are a wide range of different NO donors although only a few are in clinical use, and their chemistry and biological applications have been reviewed extensively elsewhere (231). The following text aims to present aspects of some clinically relevant NO donors and inhaled NO, that have been tested in pulmonary embolism.

#### *Organic nitrates*

The use of one organic nitrate, nitroglycerin, in angina pectoris was described in 1879 as well as its similarity to the organic nitrite, amyl nitrite (161, 166). Indeed they are very efficient anti-anginal and anti-ischemic agents, with the advantage that they rarely cause coronary steal phenomenon (221). Nitroglycerin and isosorbide mononitrate are used in acute and chronic anginal pain respectively and nitroglycerin may be used in the treatment for anal fissure (149). Isosorbide dinitrate together with hydralazine is used for heart failure in African-Americans (149). Other uses of nitroglycerin include left ventricle failure, and systemic blood pressure regulation in acute hypertensive crisis and in controlled hypotension in surgery (166). Organic

nitrates are NO donor compounds that contain a C-O-NO<sub>2</sub>-group. The mechanism of action is due to the release of NO, as shown *in vivo* by the use of exhaled NO (13, 98, 173), and the vasodilation is exerted via activation of soluble guanylate cyclase (18, 159). Several enzymes have been suggested to take part in the bioactivation of nitroglycerin and other organic nitrates, but hitherto the most convincing suggestion is mitochondrial aldehyde dehydrogenase (38). Interestingly, mice lacking the gene for this enzyme are partly resistant to nitroglycerin, but still responsive to higher doses (100 times) indicating the existence of high affinity and low affinity bioactivation pathways (37). Still the NO species that arise from bioactivation and activate guanylate cyclase are elusive, e.g. NO, inorganic nitrite or others (139). A significant clinical problem with organic nitrates is the rapid tolerance development (tachyphylaxis), especially in intravenous nitroglycerin treatment which can be studied experimentally (14, 23, 98, 220). There is also cross-tachyphylaxis between several organic nitrates (13). Probably the tolerance to nitroglycerin is due to decreased bioactivation or bioavailability of nitroglycerin-derived NO or a related species, consistent with the findings that nitroglycerin-tolerant rabbits show decreased NO generation in exhaled gas from nitroglycerin (13, 14). Several hypotheses regarding the tolerance development have been proposed, including the oxidative stress hypothesis meaning that nitroglycerin-derived NO bioavailability is reduced due to the reaction with superoxide (139). The vascular thiol depletion hypothesis is based on the interpretation that nitroglycerin is bioactivated by thiols and that nitroglycerin consumes the thiols in the cell thus limiting its own bioactivation (139). Interestingly, mice that lack mitochondrial aldehyde dehydrogenase, and therefore bioactivate nitroglycerin by the low affinity pathway, and wild type nitroglycerin-tolerant mice respond similarly to nitroglycerin, indicating that tolerance is due to inactivation of the high-affinity bioactivation pathway (37). It is suggested that bioactivation renders the mitochondrial aldehyde dehydrogenase in an inactive state and that the enzyme needs to be reduced to its initial state by a reducing agent, which may be depleted thus explaining the thiol-depletion hypothesis and the beneficial effects of antioxidants in nitroglycerin-induced tolerance (139). Therefore by using NO donors that do not need this bioactivation step or possibly utilise another enzyme system for their activation, tolerance development may be avoided.

### *Organic nitrites*

The use of one organic nitrite, amyl nitrite, in angina pectoris was first described in 1867 (30), but amyl nitrite was later replaced by nitroglycerin due to its longer duration of action (161, 166). Nowadays inhaled amyl nitrite is sometimes used as an antidote in life-threatening cyanide intoxication (127). In contrast to organic nitrates, organic nitrites exhibit little or no tolerance *in vivo* and *in vitro* and do not have substantial cross-tachyphylaxis with organic nitrates (23, 122). In healthy humans inhaled amyl nitrite produces a very rapid (within 15-20 s) systemic hypotension and reflex tachycardia (154). Inhaled amyl nitrite has shown beneficial effects in a porcine model of pharmacologically induced acute catastrophic pulmonary hypertension, but methaemoglobin was not measured (158). Inhaled ethyl nitrite has even been suggested as an inhaled therapeutic in newborns with persistent pulmonary hypertension, however methaemoglobin concentration increased to 3.2% (mean) after

4 hours of treatment and up to 8% in one newborn (156). It is suggested that inhaled ethyl nitrite is effective in reacting with and replenishing S-nitrosothiols and therefore more beneficial compared with inhaled NO in reducing pulmonary vascular resistance in a porcine model of pulmonary hypertension, however methaemoglobin may have reached 2% (157). These data show that organic nitrites are potent vasodilators with rapid on-set both in the pulmonary and systemic circulations where, seemingly, inhaled ethyl nitrite is more selective for the pulmonary circulation. The use of organic nitrites are associated with adverse effects and, specifically, the long-term use of ethyl nitrite and other organic nitrites has been questioned due to the significant methaemoglobin formation (1, 127). Other problems with organic nitrites are development of internal pressure in ampoules (“poppers”) and degradation during dilution, problems which are solved in the present thesis work. Organic nitrites are compounds that contain the C-O-N=O-group. The mechanism of action of organic nitrites are thought to be due to the release of NO and it has been demonstrated by using exhaled NO that organic nitrites are converted to NO *in vivo* (35). It has been suggested that cytosolic enzymes bioactivate organic nitrites (122). In particular, glutathione transferases have been shown to catalyse the formation of S-nitrosoglutathione from organic nitrites (15, 105, 147), which may be a step in its bioactivation. Interestingly lung, heart and liver in rats show the highest capacity of enzymatic formation of S-nitrosoglutathione from organic nitrites (15). It has also been suggested that xanthine oxidase reduces organic nitrites to NO in anaerobic conditions (63). Taken together, organic nitrites may be useful NO donors due to their rapid onset, absence of tolerance development and high abundance of enzymatic activity in lung and heart, tissues which may be primary targets for therapy. Methaemoglobin is probably the most detrimental adverse effect and should therefore be monitored.

There are several methods for producing organic nitrites, including reaction of alcohols with nitrous acid or other nitrosating agents such as nitrosyl chloride and nitrosonium salts (167, 238) or nitrosyl exchange between an alcohol and an alkyl nitrite (65). Grossi & Strazzari (86) produced cycloalkyl nitrites from the reaction of NO gas with parent cycloalkyl alcohols in the presence of oxygen. They suggested that the reaction in this situation is not through the NO molecule itself but rather from nitrous anhydride (N<sub>2</sub>O<sub>3</sub>), a known nitrosating agent, which is formed in the reaction between NO and molecular oxygen ( $4 \text{ NO} + \text{O}_2 \rightarrow 2 \text{ N}_2\text{O}_3$ ). The present thesis work introduces a method involving usage of pure nitric oxide gas for producing stable aqueous solutions of organic nitrites suitable for intravenous infusion.

#### *Sodium nitroprusside*

Intravenous infusions of sodium nitroprusside have since long been used to reduce systemic arterial blood pressure in hypertensive crises since it is a powerful vasodilator (232). However it is associated with tolerance development (232). Sodium nitroprusside is a metal-NO complex (231). In solution it is extremely sensitive to light and decomposes rapidly (231). The bioactivation of sodium nitroprusside is not fully understood and may be both enzymatic and non-enzymatic but it involves release of cyanide (231). Therefore it is associated with risk of cyanide intoxication and its use is sometimes discouraged (232).

### *Inhaled NO*

Administering vasodilators as inhalations are one option to try to confine the vasodilatory effects within the pulmonary circulation. The hemodynamic effects of inhaled NO are specific to the pulmonary circulation and it is effective in counteracting pulmonary hypertension (74) although it may also have other potentially beneficial systemic effects (48). Another important feature with inhaled NO is that it will increase perfusion in well-ventilated lung parts, thereby potentially improving arterial oxygenation (181). Inhaled NO is used in persistent pulmonary hypertension of the newborn (41) whereas use of inhaled NO in acute lung injury and acute respiratory distress syndrome is not recommended (8). The site of pulmonary vasodilation by inhaled NO has been estimated to smaller arteries and veins in contrast to an intravenous NO donor that also affects larger vessels in the lung (146, 191). Interestingly, it has been suggested that inhaled NO reaches the pulmonary vasculature to a smaller degree in non-responders of newborns with persistent pulmonary hypertension compared with responders (168). Nevertheless, inhaled NO is a very efficient therapy in some pulmonary diseases but in others it may not be effective since it may not reach upstream targets in the pulmonary circulation. In these cases an intravenous lung-selective NO donor would be preferable.

### *Inorganic nitrite*

Inorganic nitrite when infused intravenously can at high infusion rates lead to NO donation in the lungs as evidenced from measurements of exhaled NO (12) and inorganic nitrite is a potential NO donor in a multitude of situations (132). Indeed, inorganic nitrite has been shown to be a vasodilator in the pulmonary circulation (57) and therefore comparisons between inorganic nitrite and organic nitrite NO donation are relevant and will be made in the present work.

## **1.2. PULMONARY EMBOLISM**

### **1.2.1. Epidemiology of pulmonary embolism**

Pulmonary embolism is a complication of deep venous thrombosis, and these diseases are grouped as venous thromboembolism. Venous thromboembolism is a common disease affecting both sexes, with an annual incidence of 0.7-1.1 per 1000 in the general Caucasian population (199, 236). Pulmonary embolism is evident in one third to one half of the total cases of venous thromboembolism (236). The prevalence of acute pulmonary embolism in hospitalized patients has been estimated to 1% (207). Pulmonary embolism may be a complication to malignancies and other chronic diseases, but it will also occur frequently in conditions that may strike otherwise healthy people in working age and importantly the incidence of venous thromboembolism increases exponentially as early as after the age of 40 (17, 199, 236).

### **1.2.2. Diagnosis of acute pulmonary embolism**

The symptoms of pulmonary embolism are dyspnea, chest pain, palpitations, syncope, cough and haemoptysis (82, 151, 208). The signs of pulmonary embolism are tachycardia, crackles, unilateral leg swelling, cyanosis and neck vein distension (151,

208). Both the symptoms and signs of pulmonary embolism are non-specific with several differential diagnoses, thus making pulmonary embolism a true diagnostic challenge (151, 208). Normally, but not exclusively, pulmonary embolism is associated with arterial hypoxemia, and hypocapnia due to hyperventilation (151, 208). Simple prediction models such as Wells' score (235) or the revised Geneva score (128) can be used clinically to approximate the probability for pulmonary embolism and thus to estimate the need for further investigations (224). D-dimer may be used together with clinical prediction models to exclude PE but due to its low positive predictive value increased D-dimer is not specific enough (224). Multi-detector computed tomography is nowadays becoming a standalone investigation both for excluding and confirming pulmonary embolism, which is accurate if the clinical probability of pulmonary embolism is concordant (224). Right ventricle echocardiography can be used to monitor the degree of right ventricular dysfunction (82, 224). The European Society of Cardiology's guidelines suggest, that in suspected pulmonary embolism with systemic hypotension and/or shock, computed tomography should be performed immediately if possible (224). In suspected pulmonary embolism with stable hemodynamics, the guidelines suggest that the susceptibility should be estimated (with e.g. Wells' score) and in the cases where the susceptibility is low, a negative D-dimer excludes pulmonary embolism (224). If the D-dimer is positive or the susceptibility of pulmonary embolism is high, a multi-detector computed tomography should be performed (224). Despite the use of these algorithms, more than 50% of the patients that are investigated with multi-detector computed tomography will actually not have pulmonary embolism (235), meaning that approximately 50% of the computed tomographies performed are strictly speaking not necessary. They will find other diseases, e.g. pulmonary oedema due to left heart failure or pneumonia, and may therefore be of value in explaining the patient's symptoms, but these diagnoses do not require computed tomography, instead chest X-rays are sufficient. Therefore, in order to reduce the number of expensive and time-consuming investigations with computed tomography, it would be of great value to improve these clinical prediction models with new markers of acute pulmonary embolism. In the present thesis work, it was investigated in experimental models of acute pulmonary embolism if exhaled NO may be a marker identifying embolism.

### **1.2.3. Outcome and risk stratification in acute pulmonary embolism**

At presentation, most patients with pulmonary embolism are symptomatic and hemodynamically stable, whereas approximately 5-10% are in shock (82, 110). Although hemodynamically stable, almost 50% show signs of right ventricular dysfunction, which is a strong predictor of poor outcome probably because 10% develop shock later (82, 110, 119, 124). Normally right ventricular dysfunction is determined with transthoracic echocardiography but it may also be estimated with multi-detector computed tomography or laboratory tests such as cardiac troponins and natriuretic peptides (i.e. N-terminal-probrain natriuretic peptide and brain natriuretic peptide), however the laboratory tests must be interpreted carefully due to the low specificity (119, 124). The overall 30 day mortality of treated pulmonary embolism cases is estimated to approximately 12% (236), but the majority of deaths is in non-diagnosed cases (49). Ten percent of symptomatic pulmonary embolism cases are

fatal within the first hours (110), which accounts for two thirds of the mortality in recognised pulmonary embolism (241). The 3-months mortality in hemodynamically stable patients may be 10-15% and in hemodynamically un-stable patients up to 25-50% (82, 110, 241). This further strengthens the need of new and fast methods in pulmonary embolism diagnosis, the need to initiate treatment immediately and the need for new treatment methods. In order to improve the risk prediction of mortality in pulmonary embolism and to identify patients that need intensive treatment, several scoring systems, using easily available clinical data and routine laboratory tests, have been developed (224). In the guidelines of the European Society of Cardiology on pulmonary embolism it is suggested that the presence of systemic hypotension decides if the patient is of high risk (>15% early mortality) or of non-high risk (early mortality 0-15%) of early mortality (224). In the non-high risk group, an intermediate risk group (early mortality of 3-15%) is identified on the basis of right ventricular dysfunction (either by echocardiography or biochemical) and/or myocardial ischemia (biochemical markers) (224). Nevertheless, there is a clinical need for new markers that estimate the severity of pulmonary embolism that can be used in combination with the present markers in order to facilitate the decision on treatment with e.g. thrombolysis in acute pulmonary embolism. In the present thesis, it was investigated in experimental models of acute pulmonary embolism if exhaled NO may be a marker of severity of the disease.

#### **1.2.4. Treatment of acute pulmonary embolism**

The treatments of acute pulmonary embolism include hemodynamic and respiratory support, thrombolysis, surgical embolectomy, percutaneous catheter embolectomy and fragmentation and anticoagulation, depending on the severity of the pulmonary embolism (224). The guidelines suggest that patients in the high risk group should be treated with thrombolysis, if no absolute contraindications are present (risk of major bleeding), due to a significant reduction of death and recurrent pulmonary embolism in these patients (224, 230). Initially, hemodynamic and respiratory support may be a major part of the treatment in these patients (224). Surgical (preferred) or percutaneous catheter (second choice) embolectomies can be performed in high-risk patients if there are absolute contraindications to thrombolysis or if thrombolysis fails to improve hemodynamics (224). In patients with low risk of in-hospital death treatment with anticoagulation is sufficient (224). Major bleeding, e.g. intracranial hemorrhage, the feared complication of the treatment, is more frequent in patients treated with thrombolysis compared to anticoagulation (82) and happens in approximately 13% of patients treated with thrombolysis (224). Therefore it is always a risk-benefit situation for the individual patient when deciding on whether to treat with anticoagulation or thrombolysis. Still there is uncertainty about which patients in the intermediate-risk group that will actually benefit from more intensive treatments (e.g. thrombolysis). It has been suggested that thrombolysis should be considered in hemodynamically stable patients with right ventricular dysfunction shown by echocardiography and raised cardiac troponins or natriuretic peptides, however no such clinical study exists at present (119, 124-126). New markers for risk stratification of disease severity may help in this decision.



### **1.2.5. Pathophysiology of acute pulmonary embolism**

The pathophysiology of acute pulmonary embolism is pulmonary hypertension and gas exchange disturbances, with risk of right ventricular failure, circulatory collapse and death.

Pulmonary gas embolism is a special form of acute pulmonary embolisation and although the cause and treatment differ significantly from pulmonary embolism with solid emboli, the pathophysiology is rather similar (204). Pulmonary gas embolism also produces the pathological consequences that will be discussed below, but one important difference exists, it will resolve by itself within approximately 30 min (94).

#### *Pulmonary hypertension*

The pulmonary hypertension in pulmonary embolism results from both mechanical obstruction and pulmonary vasoconstriction (200, 209), although the balance between them remains to be established. There are several evidences that pulmonary vasoconstriction is important: Mechanical occlusion of the blood flow to one lung (50%) by an intravascular balloon does not increase the pulmonary arterial pressure as much as embolization of a smaller part of the lung (28, 108, 141). In previously healthy humans suffering from acute pulmonary embolism, vascular obstruction above 25-30% increases mean pulmonary arterial pressure above 20 mmHg and if the obstruction reaches 50% the mean pulmonary arterial pressure exceeds 35 mmHg (140, 141). Although significant, poor correlations were found between the extent of angiographic vascular obstruction and pulmonary arterial pressure and arterial partial pressure of oxygen in acute pulmonary embolism (140, 141). In hemodynamically stable patients with moderate pulmonary embolism, the right ventricular dysfunction does not correlate well to perfusion abnormalities in the lung (150). In cross-circulatory experiments in sheep, a transmissible factor from the animal suffering from acute pulmonary embolism induced pulmonary hypertension, decreased lung compliance and caused hyperventilation in the recipient sheep (91). Platelets are key players in acute pulmonary embolism, as they are activated on the pulmonary emboli and subsequently release several vasoconstrictors and other substances (40, 202, 223). The most important vasoconstrictors in acute pulmonary embolism are thought to be serotonin, thromboxane A<sub>2</sub> and endothelin-1 (22, 188, 193, 227). It was suggested already 50 years ago that pulmonary vasoconstriction, induced by serotonin release from platelets, contributes to the hemodynamic response in acute pulmonary embolism (43). Serotonin is released immediately after experimental pulmonary embolisation and is suggested to mediate part of the pulmonary hypertension in acute pulmonary embolism (227). In a rabbit model of acute pulmonary embolism, thromboxane A<sub>2</sub> was released immediately after embolisation and the level of increase correlated with risk of early mortality (188). In a canine model of acute pulmonary embolism, thromboxane A<sub>2</sub> was released and pretreatment with a thomboxane synthesis inhibitor abated the cardiopulmonary effects of acute pulmonary embolism (226). In pulmonary embolism endothelin-1 is released from several cells including the injured pulmonary endothelium (22). In humans suffering from pulmonary embolism and in a sheep model of pulmonary gas embolism levels of endothelin-1 were increased and in the animal model endothelin receptor antagonism

partly decreased the pulmonary hypertension (152, 203). There are also other potential vasoconstrictive mediators, although the roles of these are less defined in acute pulmonary embolism (e.g. adenosine diphosphate, platelet-derived growth factor, platelet-activating factor, angiotensin II, histamine and thrombin) (209).

*The gas exchange disturbances in acute pulmonary embolism*

The gas exchange disturbance seen in acute pulmonary embolism is characterised by four changes. First, there is an increase in high V/Q ratios and in dead-space ventilation due to mechanical obstruction of blood flow and pulmonary vasoconstriction (31, 50, 81, 102). Second, there is an increase in low V/Q ratios due to the redistribution of blood flow from obstructed parts of the lung to perfused parts of the lung (50, 81, 102). Third, a true shunt may develop as a result of atelectases caused by loss of surfactant or hemorrhage (50). Fourth, as a result of the two first changes, normal V/Q ratios are decreased (81, 102). In addition, the work of breathing may be increased due to bronchoconstriction, probably caused by mediators released from platelets (68, 87, 222) and due to decreased compliance of the lung (42, 68).

The resultant arterial hypoxemia is caused by several factors: 1) The most important reason is the development of areas with low V/Q ratios meaning that the ventilation is not enough to fully saturate the blood flow (50, 81). 2) The development of a true shunt, i.e. venous blood mixes with arterial blood without passing ventilated parts of the lung, will severely affect arterial oxygenation (47, 68). The true shunt may be intra-cardiac, most commonly through a patent foramen ovale, or it may be intrapulmonary, in areas of atelectases caused by loss of surfactant or hemorrhage (68). Increasing inhaled oxygen concentration will not improve arterial saturation in patients with a true shunt (68). 3) If cardiac output decreases, the oxygen extraction in systemic tissues will increase to maintain normal oxygen uptake (68, 81). This will further deteriorate arterial hypoxemia by enhancing the effect of the two first causes (81).

*Right ventricular dysfunction in acute pulmonary embolism*

In acute pulmonary embolism, the pulmonary hypertension in combination with the arterial hypoxaemia constitute a major threat to the right ventricle and the survival of the patient. In previously healthy patients suffering from acute pulmonary embolism, the mean pulmonary arterial pressure never exceeds 40 mmHg since the pulmonary circulation normally is a low pressure-high flow circulation (142). Normally the right myocardium is perfused during both systole and diastole since the systemic arterial blood pressure (120/80 mmHg) is higher than the systolic right ventricle pressure (20-25 mmHg) throughout the cycle (34, 237). In severe pulmonary hypertension, when the right ventricular and the right atrial pressures are increased, the perfusion of the right ventricular myocardium will be more dependent on the diastolic systemic arterial pressure and take place mostly during diastole. Systemic hypotension either as a result of the disease state itself or from vasodilator therapy will further limit perfusion of the right myocardium, thus increasing the risk of ischemia (237). In severe pulmonary hypertension, the systole of the right ventricle will have longer duration resulting in that the diastolic phase of the left ventricle starts before the systolic phase of the right ventricle ends. This provokes a leftward movement of the

septum and then when the left ventricle contracts in systole the septum moves rightwards, known as septal dyskinesia (34, 104, 237). If acute cor pulmonale develops (a dilated right ventricle), the increased volume in the right ventricle will provoke even more bulging of the septum into the left ventricle, which restricts the filling of the left ventricle in diastole (25, 104). As a consequence of septal dyskinesia and the impaired filling of the left ventricle, the left heart function is impaired which may result in systemic hypotension and decreased cardiac output (25, 34, 237). Severe dilatation of the right ventricle may also result in tricuspid regurgitation, which may decrease cardiac output even further (34, 237). The changes in right and left heart function is restored after successful treatment (104).

### **1.2.6. Vasodilator therapy in acute pulmonary embolism**

Since relieving the afterload of the right ventricle is essential, i.e. decreasing the pulmonary hypertension, in acute pulmonary hypertension several vasodilator strategies have been investigated in experimental models of acute pulmonary embolism and in a few small studies or case reports in humans (200). Vasodilator therapy in acute pulmonary embolism must fulfil several criteria: 1) preferentially be a pulmonary vasodilator and not induce systemic hypotension since that will decrease the oxygenation of the right heart; 2) maintain normal cardiac output, increase blood flow in partly obstructed well-ventilated lung parts and therefore decrease blood flow in lung parts with low V/Q ratios, in order to maintain or improve pulmonary gas exchange; 3) decrease the oxygen consumption of the right heart. When considering the vast range of vasoconstrictors involved or potentially involved in acute pulmonary embolism, solely receptor antagonist treatment of one constrictor should not be expected to be sufficient. Therefore the present thesis work concentrated on investigating vasodilator compounds in pulmonary embolism.

#### *Intravenous vasodilators*

In the first randomised controlled trial of vasodilator treatment in humans suffering from acute pulmonary embolisation, prostaglandin I<sub>2</sub> infusion intravenously did not decrease right ventricle dysfunction or affect the increased pulmonary arterial pressure (121). In contrast, in pulmonary embolisation with autologous blood clots in dogs, prostaglandin I<sub>2</sub> decreased pulmonary hypertension and improved gas exchange paralleled with a reduction of systemic arterial blood pressure by 30 mmHg (226). In a porcine model of pulmonary microembolism nitroglycerin and sodium nitroprusside intravenously had some effects on the increase in pulmonary vascular resistance but they decreased the systemic vascular resistance relatively more (143). In contrast to prostaglandin E<sub>1</sub> that showed better selectivity towards the pulmonary circulation but increased the shunt fraction. Hydralazine showed even more selectivity towards the systemic circulation than nitroglycerin and nitroprusside (143). In a canine model of acute pulmonary embolism, nitroglycerin (in a 10-fold lower dose) did not affect pulmonary and systemic vascular resistances but impaired the gas exchange (183). In a lamb model of pulmonary gas embolisation, nitroprusside had moderate beneficial effects on pulmonary vascular resistance but the vasodilatory effects were more pronounced in the systemic circulation (72). In a canine model of pulmonary embolisation with autologous blood clots, hydralazine and nitroprusside decreased

pulmonary vascular resistance however the effect on systemic circulation was larger whereas prostaglandin E<sub>1</sub> only induced systemic hypotension (52). In 10 humans suffering from pulmonary embolism intravenous infusion of ketanserin (a serotonin receptor 2 antagonist) slightly decreased pulmonary arterial pressure and pulmonary vascular resistance (-16%), together with a 10% reduction in systemic vascular resistance (96). The modest effect of the serotonin antagonist may be explained by the delay between the pulmonary embolisation and the trial (1-13 days) and that in the human pulmonary circulation it is the serotonin receptor 1D that triggers vasoconstriction (133). In dogs where the lungs were embolised with blood clots, ketanserin decreased the pulmonary hypertension and improved gas exchange without untoward systemic affection (99). These studies show that NO donors and other vasodilators are able to dilate the pulmonary circulation after pulmonary embolism, thus further strengthening the belief that active pulmonary vasoconstriction is part of the pathophysiology of pulmonary embolism, even in humans. They also show that intravenous vasodilators are associated with risks of systemic hypotension and worsening of pulmonary gas exchange and that creating intravenous vasodilators with specificity to the pulmonary circulation may be a challenge.

### *Inhaled agents*

Inhaled prostaglandin I<sub>2</sub> in a human suffering from massive pulmonary embolism slightly decreased the pulmonary hypertension and improved pulmonary gas exchange (234). The use of inhaled NO (5-50 ppm) in humans suffering from massive pulmonary embolism has been described in a few case reports and shows mostly moderate decreases in pulmonary hypertension and improved pulmonary gas exchange (214, 219). In pulmonary microembolism in dogs inhaled NO (50 ppm) attenuated the increase in pulmonary vascular resistance by 40% and slightly improved pulmonary gas exchange, whereas inhaled prostaglandin I<sub>2</sub> had no effects (248). In a porcine model of acute massive pulmonary microembolism, inhaled NO (5-80 ppm) reduced by 25% the increase in pulmonary vascular resistance and decreased platelet aggregation in the lung, without improving pulmonary gas exchange (27, 84). In pulmonary gas embolism in dogs, NO inhalation (3 ppm) lowered 25% of the increase in pulmonary vascular resistance and decreased the release of thromboxane A<sub>2</sub>, whereas inhaled NO of 40 ppm did not have any additional effect, however gas exchange was slightly impaired (218). In a canine model of autologous clot embolisation, the combination of inhaled NO (40 ppm) with an endothelin receptor A antagonist was only slightly more beneficial than inhaled NO alone (129). These studies show that especially inhaled NO in acute pulmonary embolisation attenuates part (up to 40%) of the increase in pulmonary vascular resistance without systemic hypotension; however the effects on pulmonary gas exchange were conflicting. In the present thesis work it is suggested that more than 40% of the pulmonary hypertension may be reversed by a selective intravenous pulmonary vasodilator, since the intravenous administration allows the NO donation to reach larger vessels that might dominate in the vasoconstrictive process.

### **1.3. NITRIC OXIDE IN PULMONARY EMBOLISM**

Before this thesis project was initiated it was known that fatal venous gas infusions increased exhaled NO (88), that pulmonary gas embolism in hemodiluted animals resulted in increased expired NO output (51), and that wedging of a pulmonary catheter and thus obstructing the blood flow increased exhaled NO from these parts of the lung (71).

It was known that NO-synthase inhibition augmented platelet aggregation induced by adenosine diphosphate, platelet-activating factor or thrombin (138), reduced the dose of collagen+adrenalin required to induce thromboembolic mortality, increased bubble formation after decompression and reduced survival in decompressed rats (239). Furthermore it was known that treatment of acute pulmonary embolism with intravenous NO donors (e.g. nitroglycerin and nitroprusside) induces systemic hypotension thus limiting its use in this condition (72, 143). Inhaled NO showed promising but moderate effects on the pulmonary hypertension in acute pulmonary hypertension without inducing systemic hypotension. In contrast treatment with L-arginine in acute pulmonary embolism was not beneficial (205).

## **2. AIMS**

The overall aim of this thesis was to investigate the role of NO formation in pulmonary vascular regulation, especially during acute pulmonary embolism.

More specifically, the studies aimed to:

1. Investigate in what direction NO changes during acute pulmonary embolism and whether it relates to changes in other respiratory gases, especially carbon dioxide.
2. Investigate whether endogenous NO has salutatory or damaging functions during acute pulmonary embolism by studying the effects of NO synthase inhibition on pulmonary embolism.
3. Find means of identifying lung selective intravenous NO donors, in order to obtain efficient pulmonary vasodilation in situations with compromised pulmonary circulation.
4. Investigate the effects of intravenous lung selective NO donors in acute pulmonary embolism.

### 3. METHODS

The present thesis work employed rabbit *in vivo* models of experimental pulmonary hypertension and pulmonary embolism in order to investigate the role and response of endogenous NO and to investigate the therapeutic potential of new NO donors, in these conditions. A rabbit *in vivo* bioassay model was used for determining *in vivo* NO donating capabilities and selectivity of NO donor solutions. A new production method for producing solutions of new NO donating organic nitrites suitable for intravenous infusions was invented. A method where HPLC was connected to on-line detection and quantification of inorganic and organic nitrites was developed. Gas chromatography-mass spectrometry (GC-MS) was used for chemical identification of some new organic nitrites. Molecular properties of the used organic nitrites were calculated and were used in order to understand critical properties of the individual organic nitrite molecules for selectivity towards the pulmonary and systemic circulation respectively. For a detailed description of the methodology used in this thesis work, please refer to the individual paper.

#### 3.1. NO DONOR SOLUTIONS

##### 3.1.1. Preparation of NO donor solutions for intravenous infusions and chemical characterisation (Paper III, IV)

Aqueous solutions of alcohols and sugars and related compounds (Table 1), saline and a lipid emulsion were prepared and placed in gas tight cylinders (volume 20-150 ml) with taps in both ends and a sideway port with a septum plug in the middle portion. The cylinders were filled to 75% with solution, allowing a gaseous head-space. The solutions were deoxygenated by means of helium bubbling for 10 min and purged with pure nitric oxide gas for 3-5 min. From the gas-tight cylinders the solutions were transferred via the side port septum plug to syringes for i.v. infusions in rabbits for determination of NO donating capabilities. Samples were also analysed by GC-MS for chemical identification, and either directly in a nitrite reduction system or in HPLC coupled to nitrite reduction for NO donating capability and for quantification of the content of NO gas and inorganic and organic nitrites.

##### 3.1.2. HPLC coupled to on-line measurement of NO/nitrites by chemiluminescence (Paper III, IV)

Samples of NO donor solutions were analysed in HPLC, HPLC coupled to on-line measurements of NO/nitrites (denoted LC-NO) and directly in the nitrite reduction system with or without reducing agent.

###### *The HPLC system*

The HPLC consisted of equipments stated in paper III and IV and in Nilsson *et al.* (165). Briefly, a reverse phase HPLC was used, with analytical C<sub>18</sub> or CN columns eluted either in isocratic mode (5-10 mM ammonium formate in water with 0-8% of acetonitrile) or with a linear gradient (from 5-10 mM ammonium formate in water followed by increasing proportions [3%/min] of methanol or acetonitrile) at flow rates of 0.5-1.0 ml min<sup>-1</sup> depending on the size of the column. The UV absorbance during

elution was measured with a variable UV absorbance detector at 227 nm or 254 nm and a scanning UV absorbance detector (190 – 370 nm). Pumps were controlled and UV absorbance measurements were collected by commercial hardware and software. The volume of the injector loop was 2.2 ml and samples, nitrite standards and commercially available organic nitrites were injected at volumes of 2  $\mu$ l - 2 ml. In some experiments in order to enrich the compounds of interest for GC-MS, repeated injections were performed when the retention times of these compounds allowed. The effluent from the HPLC system could then either be collected in gas tight syringes for GC-MS or continuously be introduced into a nitrite reduction system for on-line measurement of NO/inorganic nitrite/organic nitrites by means of a 1/16 inch PolyEtherEtherKetone (PEEK) capillary.

*On-line measurement of NO/inorganic nitrite/organic nitrite by chemiluminescence*

The NO/nitrites analysis utilised a similar system as in Hallén *et al.* (90), based on the method by Walters *et al.* (229). Inorganic and organic nitrites are reduced to NO in this environment whereas nitrates are not reduced (229). Aliquots or the effluent from the HPLC were intermittently respectively continuously injected in a temperature-controlled reaction chamber (total volume 300 ml) containing 75 ml of 1% (w/v) sodium iodide in hot (89° C) glacial acetic acid. In some experiments the reducing agent was replaced by ultra-pure water in order to measure the content of NO gas in the samples. The reaction solution was purged with N<sub>2</sub>-gas (150-200 ml min<sup>-1</sup>) to deoxygenate the solution and to carry NO gas to a chemiluminescence NO analyser system. In the NO analyser ozone was produced in an oxygen flow of 100 ml min<sup>-1</sup> and made to react in a vacuum chamber with the NO in the incoming gas from the reducing chamber (sample flow rate 150-200 ml min<sup>-1</sup>). The signal from the photomultiplier tube was collected by a computerised data acquisition system. The system was intermittently calibrated with aliquots (10-500  $\mu$ l) of known amounts of sodium nitrite either by direct injection or via the HPLC system (detection limits: 5 pmol and 10 pmol respectively). When only measuring NO gas with the system, it was calibrated by direct injections of known amounts of NO gas in nitrogen. The sensitivity of the photomultiplier tube was adjusted in order to measure in the linear range. In experiments where the HPLC effluent was introduced into the reaction chamber, the volume in the reaction chamber increased slowly and intermittent withdrawal and refurbishment of liquid was necessary. In order to minimise the interference of different volumes in the chamber, calibration was always done before and after each analysis. These calibrations were very similar and the mean between the two sets was used in the calculation of quantity. Quantification of NO content was made by comparing peak area measurements with the peak area of a known amount of standard. The recovery of the LC-NO system was 88-100%.

**3.1.3. Gas chromatography-mass spectrometry (Paper III)**

Eluted peaks from the HPLC with the reaction mixture of propanol and NO gas were collected in gas tight syringes and extracted by an equal volume of toluene if necessary. Alternatively, samples of 25-100 % propanol and NO gas and samples of commercial propyl nitrite (for comparisons) diluted in toluene were injected directly in the gas chromatography column thus bypassing the extraction procedure. Fractions



were injected at 1  $\mu\text{l}$  onto an Agilent DB-5MS gas chromatography column (0.25 mm i.d., 30 m length, 1  $\mu\text{m}$  phase thickness). In addition, 0.5  $\mu\text{l}$  samples of deoxygenated 25-100% 1,2-propanediol treated with NO gas were injected in the GC-MS, now equipped with an Agilent DB-1701 column (0.25 mm i.d., 30 m length, 0.25  $\mu\text{m}$  phase thickness), in a total He flow of 30  $\text{ml min}^{-1}$ , split fraction 1:5. In all analyses, the initial temperature was 40° C and was increased by 10° C  $\text{min}^{-1}$  until a temperature of 200°-250° C was reached. Mass spectrometry was either performed in electron impact mode, or in chemical ionisation mode using a chemical ionisation interface supplied with a methane flow. Detection of eluted compounds was made by an Agilent 5973 Mass Selective Detector. Data were collected and analysed with computer software and a library of mass spectra.

### **3.2. RABBIT IN VIVO MODEL (PAPER I-IV)**

#### **3.2.1. General procedure (Paper I-IV)**

Rabbits (New Zealand White, 2.0-3.5 kg) were anaesthetised with pentobarbital sodium (40-70  $\text{mg kg}^{-1}$  body weight) intravenously through an ear vein. Mechanical ventilation (respiratory rate 40  $\text{min}^{-1}$ ) with charcoal-filtered air was achieved by tracheostomy, insertion of a tracheal cannula and a rodent volume-controlled ventilator. The expiratory gas flow was altered between a lower positive end expiratory pressure (PEEP, 9 min, 1-2  $\text{cm H}_2\text{O}$ ) and a higher PEEP (1 min, 4-6  $\text{cm H}_2\text{O}$ ) in order to optimise ventilation and to prevent formation of atelectasis. Insufflation pressure, tidal volume, and  $\text{CO}_2/\text{O}_2$  in inspiratory and expiratory gases were measured at side arms of the tracheal cannula or by a Datex Pedi-lite+ flow sensor and gas sampler immediately after the tracheal cannula. Nitric oxide in mixed exhaled gas (FENO) was continuously measured by a chemiluminescence-based system sampling at 100-140  $\text{ml min}^{-1}$  through a Naphion sampling catheter at the end of a mixing chamber (size: 5 tidal volumes) connected to the ventilator exhaust. Tidal volume was adjusted to attain an end-tidal  $\text{CO}_2$  concentration of 4.5-5.5% at baseline. Body temperature of 38-39° C was maintained by use of a heating pad and a hot-water heated bench. During the experiments the animals received a continuous intravenous infusion, via an ear vein, of glucose (24-26  $\text{g l}^{-1}$ ), dextran 70 (27-28  $\text{g l}^{-1}$ ) and sodium bicarbonate (6.2-6.6  $\text{g l}^{-1}$ ) to compensate for fluid loss and to maintain an adequate blood acid-base status. Pentobarbital sodium was added to the infusate (2.1-4.2  $\text{g l}^{-1}$ ) to maintain anaesthesia. In paper I and II the animals were paralysed with addition of pancuronium bromide to the infusate (98  $\text{mg l}^{-1}$ ). The infusion rate was administered at a rate of 5 or 10  $\text{ml kg}^{-1} \text{h}^{-1}$  depending on the extent of surgery. Systemic arterial blood pressure and heart rate were measured and arterial blood samples were collected from a heparinised catheter in the left common carotid artery. Material for pulmonary embolisation and drug infusions were administered into a saline carrier flow via a catheter in the right jugular vein with the tip approximately at heart level.

### **3.2.2. Measurement of pulmonary hemodynamics and gas exchange parameters (Paper III, IV)**

The animals were prepared as above and a median sternotomy was performed. A heparinised catheter was inserted in the left jugular vein and advanced to the pulmonary artery for measurement of pulmonary arterial pressure and sampling of mixed venous blood for blood gas analysis. An ultrasonic flow probe was placed around the ascending aorta and this blood flow was considered as cardiac output. A catheter was introduced into the left atrium through a puncture wound in the left heart auricle for measurements of left atrial pressure. The open thorax was covered with plastic foil to minimise fluid loss. Synchronously collected samples of mixed exhaled gas, arterial blood and mixed venous blood were analysed and the values were used in the calculation of physiological dead-space ventilation and venous admixture (see paper IV for these and other calculations).

### **3.2.3. Experimental pulmonary hypertension (Paper III)**

Acute pulmonary hypertension was induced by means of a continuous intravenous of the thromboxane A<sub>2</sub>-mimetic U46619 (150-300 ng kg<sup>-1</sup> min<sup>-1</sup>) to reach a mean pulmonary arterial pressure of 30-35 mmHg. When a stable state of pulmonary hypertension was reached, the effects of intravenous infusions of NO donor solutions, on pulmonary and systemic hemodynamics and on blood gases, were evaluated.

### **3.2.4. Experimental pulmonary embolism (Paper I, II, IV)**

Animals were prepared and monitored according to the general procedure. In paper I, pulmonary embolism was induced by a fast intravenous infusion (500 µl min<sup>-1</sup>) of air (100 µl kg<sup>-1</sup>) in order to induce pulmonary gas embolism. In paper II and IV, acute pulmonary embolism was induced by means of an intravenous infusion of homogenised autologous skeletal muscle tissue to mimic acute pulmonary embolism of solid material and it was prepared by modification of a previously described method (183). The right anterior tibial muscle was resected and placed in saline. Visible connective tissue and blood was removed, the muscle piece was dissolved in saline to a concentration of 0.1 g muscle ml<sup>-1</sup> and homogenized. Heparin was added to the mixture (50 IE heparin ml<sup>-1</sup>), in order to prevent clotting of the solution. The homogenate was passed through a 0.5-1 mm mesh to prevent obstruction in the three-way stopcock of the venous catheter. In paper I and II some groups of animals were pre-treated with the NO synthase inhibitor N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME; 30 mg kg<sup>-1</sup>), before pulmonary embolisation, in order to evaluate the function of endogenous NO in acute pulmonary embolism. This dose of L-NAME has previously been shown to abolish FENO and to increase the pulmonary vascular resistance by 75% in rabbits (5). In paper I, CO<sub>2</sub> was added to inhaled gas of one group of animals 5 min after pulmonary embolisation in order to normalise airway CO<sub>2</sub> concentration. In paper II, several different doses of MPE (7.5-60 mg homogenised muscle kg<sup>-1</sup> body weight at 15 mg kg<sup>-1</sup> min<sup>-1</sup>) was administered to different groups with intact and inhibited endogenous NO production. The animals were followed for at least 60 min after pulmonary embolisation and in paper II the survival of each group was evaluated. In paper I and II the effect of pulmonary

embolisation was evaluated on respiratory gases including exhaled NO, blood gases and systemic hemodynamics.

In paper IV, deoxygenated NO-treated 1,2-propanediol (25% in saline, v/v, containing 9 mM of the organic mononitrites of 1,2-propanediol in Figure 6, denoted PDNO), placebo (1,2-propanediol, 25% in saline, v/v, denoted PD) or of control solution (saline) were administered intravenously 20-60 min after acute pulmonary embolisation (30 mg muscle kg<sup>-1</sup>). The dose of pulmonary emboli was determined in pilot experiments and by the results in paper II, in order to induce moderate pulmonary embolisation that was large enough to be a considerable challenge but small enough so that all animals survived the observation period. The effects were evaluated on respiratory gases including exhaled NO, blood gases, methaemoglobin formation, systemic and pulmonary hemodynamics, right ventricle rate-pressure product, physiological dead space and venous admixture (see paper IV for calculations).

### **3.2.5. Evaluation of the *in vivo* NO donating capacity and selectivity of NO donor solutions (Paper III)**

Initial experiments included infusions in rabbits of deoxygenated NO-treated saline and deoxygenated NO-treated lipid emulsion as means of administering NO gas intravenously. In further experiments, rabbits received the candidate NO donor solutions (prepared as above, Table 1) intravenously (3-300 µl kg<sup>-1</sup> min<sup>-1</sup>) into a carrier flow of saline. Each rabbit received more than one of the NO donor solutions and were allowed ample intervention-free periods between infusions to reach stable baseline conditions and to exclude cross-tolerance. Arterial blood samples were intermittently analysed for blood gases and methaemoglobin. In some experiments the endogenous NO production was inhibited with L-NAME (30 mg kg<sup>-1</sup>). The capacity of NO donation *in vivo* was determined positive if both FENO increased and mean systemic arterial blood pressure (MAP) decreased. The relative effects of the individual NO donor solution on FENO (increase in ppb from baseline) and MAP (change in % of baseline) were considered as a measure (change in % of baseline divided with increase in ppb from baseline; MAP/FENO-index) of selectivity towards the pulmonary and systemic circulation respectively.

### **3.2.6. Investigation of NO donating capabilities of the organic nitrites of 1,2-propanediol, inorganic nitrite and NO gas (Paper IV)**

PDNO, 1,2-propanediol (25% in saline, v/v) solutions with and without addition of inorganic nitrite (20 mM) and deoxygenated NO-treated saline (Saline+NO) were given intravenously to rabbits in dose-response experiments. The added amount of inorganic nitrite in the 1,2-propanediol+nitrite (PD+nitrite) solution was chosen to approximate the total content of organic plus inorganic nitrites in the PDNO solution (Table 1). The effects of the infusions on FENO, MAP, heart rate, blood gases and methaemoglobin were evaluated.

### **3.2.7. Computation of physico-chemical descriptors of the NO donor molecules (Paper III)**

The calculator plugin modules of MarvinSketch were used to obtain molecular descriptors of the NO donor molecules. These descriptors included octanol/water partition coefficient (Log P), molecular size descriptors (molecular weight, molecular surface area, molar refractivity, maximal and minimal projection radii and projection area, polarizability), molecular shape descriptors (the ratios between maximal and minimal projection radii and projection area respectively) and polar and non-polar surface area. The relative polar surface area of each donor molecule was calculated by dividing polar surface area with molecular surface area. These descriptors were then used as independent variables in multiple linear regression models using the log (-MAP/FENO-index) as the dependent variable. The best fits were determined by using the best subset function of the statistical software.

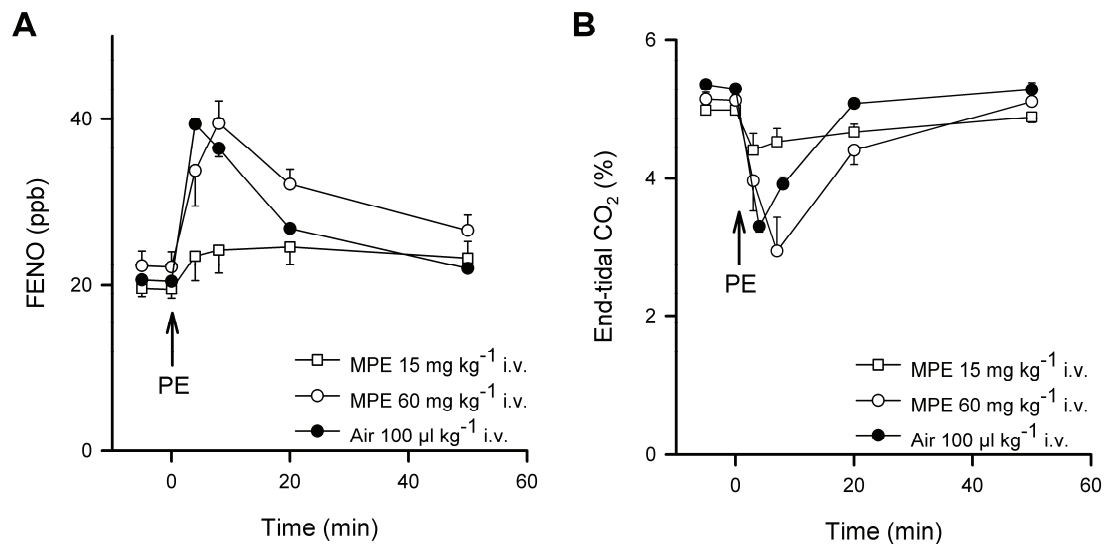
### **3.3. STATISTICAL ANALYSIS (PAPER I-IV)**

Descriptive data are presented as mean  $\pm$  standard error of the mean. Comparisons between groups were made by the student *t*-test or one way ANOVA and repeated comparisons within one group were made by a paired *t*-test or repeated measures ANOVA. Repeated comparisons between two groups were performed by two-way ANOVA on repeated measures. Survival was analysed with Fischer's exact test and survival fraction was calculated according to the Kaplan–Meier method. Linear correlations were made by using Pearson Product Moment Correlation. Multiple linear regressions were made by using the best subsets function. Models including variables with non-significant variables ( $P > 0.05$ ) or with severe multicollinearity (Variance Inflation Factor  $> 4$ ) were excluded.  $P < 0.05$  was considered statistically significant. All statistical analyses were made by means of SigmaStat (v. 3.10.0) and SigmaPlot (v. 9.01, Systat Software Inc., San Jose, CA, USA).

## 4. RESULTS AND DISCUSSION

### 4.1. EFFECTS OF PULMONARY EMBOLISATION (PAPER I, II, IV)

Acute pulmonary embolisation with both air and solid emboli in these rabbits rapidly increased FENO (Figure 1). The increase in FENO peaked within 5-10 min after pulmonary embolisation and the peak increase represented approximately a doubling of FENO. After the peak increase, FENO slowly declined towards baseline values. In pulmonary gas embolism FENO was normalised within 30-50 min. This is in agreement with that pulmonary gas embolism resolves by itself within 30 min after air infusion (94). In experiments with solid emboli the increase in FENO was still significant 60 min after severe and moderate pulmonary embolisation. The time course of the rise in FENO (fast peak followed by a slow decline) resembles the time course of the release of mediators in acute pulmonary embolism, e.g. thromboxane A<sub>2</sub> and serotonin (188, 227). In parallel to the increase in FENO in both models of pulmonary embolisation, end-tidal CO<sub>2</sub> decreased and MAP decreased while heart rate was unchanged. The arterial partial pressure of O<sub>2</sub> and CO<sub>2</sub> decreased respectively increased and arterial pH decreased, after pulmonary embolism. From the experiments with solid emboli, it is evident that these changes including the increase FENO are dependent on the dose of embolic material (Figure 1). Pulmonary embolisation increased pulmonary arterial pressure, pulmonary vascular resistance and right ventricle rate-pressure product (Figure 10). Physiological dead-space ventilation and venous admixture increased after pulmonary embolisation.



**Figure 1.** The responses of exhaled NO (panel A, FENO) and end-tidal CO<sub>2</sub> (panel B, ETCO<sub>2</sub>) to acute pulmonary embolisation with an intravenous infusion of either air (100 µl kg<sup>-1</sup> at 500 µl min<sup>-1</sup>) or autologous homogenised skeletal muscle (MPE 15-60 mg kg<sup>-1</sup> at 15 mg kg<sup>-1</sup> min<sup>-1</sup>). Data compiled from paper I and II.

*Methodological considerations on experimental models of acute pulmonary embolism*

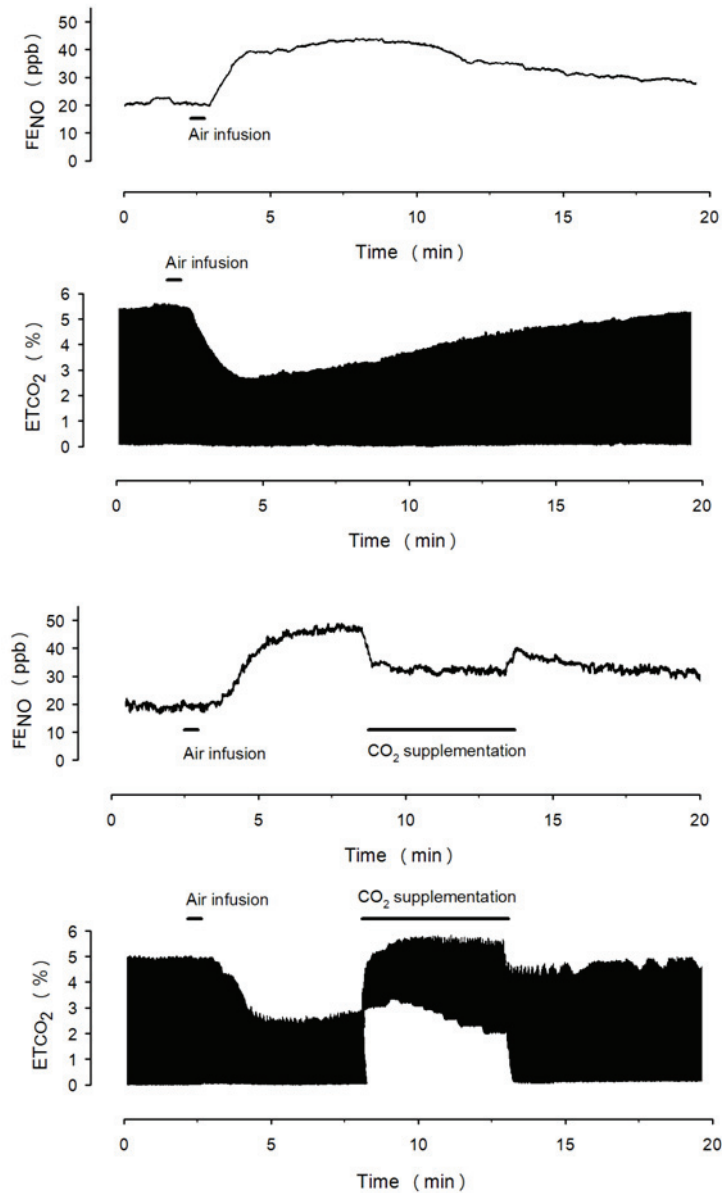
There are several ways of inducing experimental pulmonary embolism including intravenous infusion of autologous blood clots (188, 227), of gas (193), of microspheres (27) and of autologous muscle (70, 160, 183, 215-217). In the present thesis work we used gas and autologous homogenised muscle to induce pulmonary embolism to mimic pulmonary gas embolism and pulmonary thromboembolic disease in humans. Pilot experiments with intravenous infusions of coagulated and homogenised blood showed that haemoglobin release during embolisation interfered with exhaled nitric oxide measurements, i.e. free haemoglobin scavenged NO produced in the lung. In addition, intravenous infusions of coagulated blood indicated that this method was less reproducible and that the emboli slowly resolved by itself in the presence of small amounts of heparin (from the heparinised catheters). We did not use thrombin when coagulating the clot which probably would have produced more stable emboli (188, 227). Importantly, in these pilot experiments the initial response on the measured parameters (e.g. FENO, pulmonary vascular resistance and blood gases) to intravenous infusions of coagulated blood was completely similar to intravenous infusions of gas and homogenised muscle. As outlined in the introduction of the present thesis, all the changes of measured parameters, except the raised CO<sub>2</sub> in arterial blood, are consequences of acute pulmonary embolisation also in humans, thus validating that the used models of acute pulmonary embolism are representative for the disease in humans. The increase in arterial partial pressure of CO<sub>2</sub> is due to the decreased gas exchange area (i.e. increased physiological dead-space ventilation) and is a consequence of that the mechanical ventilation was not adjusted after pulmonary embolisation as it is in spontaneously breathing subjects by hyperventilation. Indeed, autologous muscle infusion is suggested to mimic the acute phase of pulmonary embolisation (183) and to produce immediate and consistent hemodynamic changes comparable with those occurring in humans with massive and diffuse PE (160).

Another factor that may affect the response to pulmonary embolisation is the size of the emboli. It might be that small emboli induce a shunt and the larger emboli induce areas of increased ventilation-perfusion ratios (53, 54). Whether such a comparison is relevant for the present studies is not known.

**4.2. EFFECTS OF CO<sub>2</sub> SUPPLEMENTATION ON EXHALED NO IN PULMONARY EMBOLISATION AND EFFECTS OF THROMBOXANE A<sub>2</sub>-MIMETIC ON EXHALED NO (PAPER I, III)**

Normalisation of airway CO<sub>2</sub> 5 min after pulmonary gas embolisation rapidly attenuated the increase in FENO by approximately 50% (Figure 2). This finding suggests that a part of the increase in FENO in pulmonary embolism is due to reduced inhibition of CO<sub>2</sub> on the pulmonary production of NO. Notably intravenous infusion of U46619, a thromboxane A<sub>2</sub>-mimetic, did not change FENO despite inducing a significant pulmonary hypertension, thus indicating that thromboxane-receptor agonism does not increase FENO (paper III). The finding that normalising CO<sub>2</sub> after pulmonary embolisation attenuates the increase in FENO is concordant with that airway CO<sub>2</sub> is a regulator of pulmonary NO production (4, 210). This effect and other

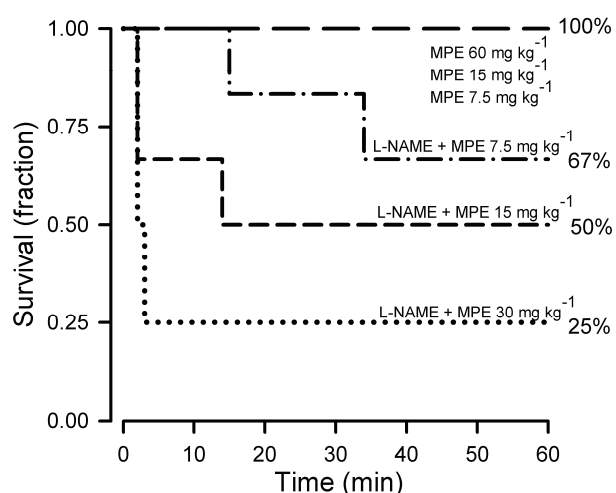
tentative mechanisms for the increase in FENO in pulmonary embolism will be discussed in section “General discussion”.



**Figure 2.** Anaesthetised and ventilated rabbits. Experimental recordings of fraction of NO in mixed exhaled gas (FENO) and end-tidal CO<sub>2</sub> (ETCO<sub>2</sub>) following intravenous bolus infusion of air (100  $\mu\text{l kg}^{-1}$  at a rate of 500  $\mu\text{l min}^{-1}$ ). Panel A, control animal; Panel B, animal where CO<sub>2</sub> was added to the inspired gas to yield ETCO<sub>2</sub> of  $110 \pm 5\%$  of pre-infusion levels. Figure 1 in paper I.

### 4.3. EFFECTS OF INHIBITION OF ENDOGENOUS NO PRODUCTION IN COMBINATION WITH ACUTE PULMONARY EMBOLISM (PAPER I, II)

Endogenous enzymatic NO production was inhibited by L-NAME ( $30 \text{ mg kg}^{-1}$ , i.v.) 30 min before pulmonary embolisation with either gas ( $100 \mu\text{l kg}^{-1}$  at a rate of  $500 \mu\text{l min}^{-1}$ ) or solid emboli ( $7.5\text{-}30 \text{ mg kg}^{-1}$ ). L-NAME treatment *per se* decreased FENO to below detection limit ( $<1 \text{ ppb}$ ) and increased MAP (by approximately  $15\text{-}20 \text{ mmHg}$ ). Heart rate decreased in some groups of animals and blood-gas values were not affected by L-NAME. It is previously known that inhibition of endogenous NO production in rabbits increases pulmonary vascular resistance and that acute inhibition of NO synthesis is well tolerated in rabbits (5, 177). Acute pulmonary embolisation with both air and solid emboli in the presence of L-NAME evoked severely augmented hemodynamic responses and blood-gas values indicating increased sensitivity towards pulmonary embolisation. All animals with intact NO production survived at least 60 min after intravenous gas infusion and solid emboli infusion (Figure 3). In contrary, all animals that were pre-treated with L-NAME died within 40 min after the venous gas infusion. Similarly, the survival of animals pre-treated with L-NAME in combination with solid pulmonary embolisation was significantly decreased (Figure 3). These results clearly show that the endogenous NO production is protective in acute pulmonary embolism. Previously shown, NOS inhibition reduced the dose of collagen+adrenalin required to induce thromboembolic mortality in mice (69). NOS inhibition has been shown to increase bubble formation after decompression and reduce survival in rats (239). Our results has also been confirmed by others in a canine model of acute pulmonary embolism with autologous blood clots (60). Attempts have also been made to define the role of NO derived from the inducible NOS, however these results are conflicting (59, 60), perhaps due to non-specific effects of the inhibitors used (164).



**Figure 3.** Anaesthetised and ventilated rabbits. The lines represent the survival proportion during the first 60 min after muscle tissue pulmonary embolisation (MPE administered at time 0) for the non-pretreated groups [long-dash line; MPE of  $60 \text{ (n=6)}$ ,  $15 \text{ (n=5)}$ , or  $7.5 \text{ mg kg}^{-1} \text{ (n=5)}$ ] and the L-NAME ( $30 \text{ mg kg}^{-1}$  i.v.) pretreated groups [MPE of  $30 \text{ (n=4)}$ ,  $15 \text{ (n=6)}$ ,  $7.5 \text{ mg kg}^{-1} \text{ (n=6)}$ ]. Figure 4 in paper II.

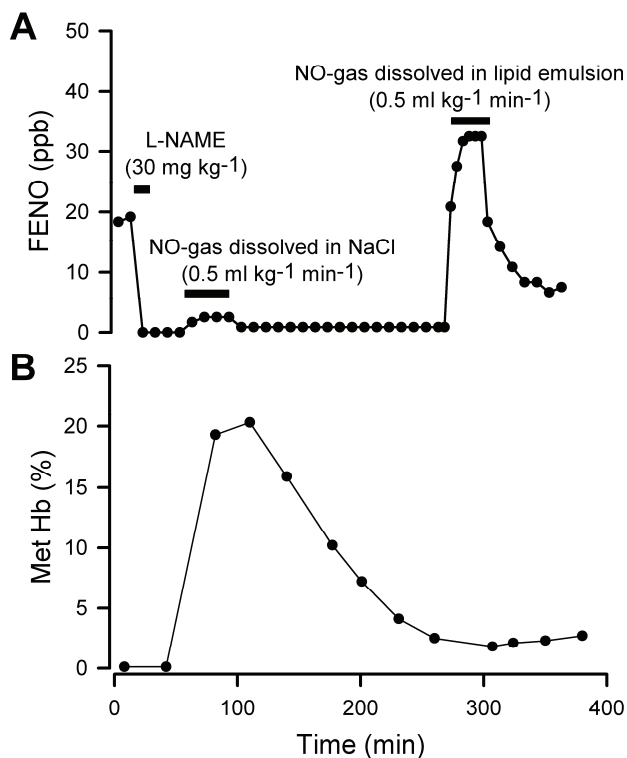


#### 4.4. FORMATION OF NEW NO DONORS BY USING NITRIC OXIDE GAS

The first part of this thesis work clearly demonstrated a protective role of the endogenous NO production in acute pulmonary embolism and therefore it was hypothesised that exogenous NO would be beneficial in this condition. It was believed that an intravenous lung-selective NO donor would be preferable since inhaled NO in acute pulmonary embolism only caused moderate decreases of the pulmonary hypertension (248) and that nitroglycerin and sodium nitroprusside caused detrimental systemic hypotension (143). Such an NO donor must have increased selectivity for the pulmonary circulation in order to avoid systemic hypotension and lack tolerance development. It was felt that the presently available NO donors did not fulfil these criteria and therefore we explored new ways of delivering NO intravenously.

##### 4.4.1. Exploration of NO donating capacity *in vivo* (Paper III)

Due to the lipophilicity of the NO molecule, it was first hypothesised that NO gas dissolved in a lipid emulsion was a way to deliver NO gas intravenously to the pulmonary circulation. Deoxygenated saline equilibrated with NO gas administered intravenously to a rabbit with inhibited NO production (L-NAME 30 mg kg<sup>-1</sup>) resulted in a small increase in FENO and a very high methaemoglobin concentration (Figure 4). In contrary, an infusion of deoxygenated NO-treated lipid emulsion at the same infusion rate in the same animal yielded a large increase in FENO whereas methaemoglobin was essentially not changed (Figure 4).



**Figure 4.** Anaesthetised and ventilated rabbit. Exhaled NO (FENO, panel A) and methaemoglobinemia (met Hb, panel B) during infusion (0.5 ml kg<sup>-1</sup> min<sup>-1</sup>) of deoxygenated NO-treated saline and deoxygenated NO-treated lipid emulsion, as indicated by horizontal bars. Endogenous NO production was inhibited (L-NAME 30 mg kg<sup>-1</sup>, indicated by a horizontal bar). Figure 1 from paper III.

**Table 1.** Overview of the formation and the identification of organic nitrites when reacting deoxygenated aqueous solutions of parent compounds with pure NO gas.

Parent compounds	NO donation <i>in vivo</i>	UV absorbance in HPLC	NO release in LC-NO	Identified by GC-MS
1-Propanol	+	+*	+ <sup>#</sup>	+
1,2-Propanediol	+	+	+	+
Glycerol	+	+	+	
Glucose	+	+	+	
Ethanol		+*	+ <sup>#</sup>	
Isobutanol	+	+*		
Butanol		+*		
Sucrose	+	+		
2-Propanol	+			
1,3-Propanediol	+			
2-Deoxyribose	+			
1-O-Methyl-2-deoxyribose	+			
Ribose	+			
6-Deoxygalactose	+			
Galactose	+			
Fructose	+			
Sorbitol	+			
Mannitol	+			
Lactobionic acid	+			
Polyethylene glycol	+			
Inulin	+			
Dextran	+			
Fuoidan	+			
2-Amino-1,3-propanediol	–			
3-Amino-1,2-propanediol	–			
Lactic acid	–			
Alanine	–			
Glycine	–			
Glucosamine	–			

NO donation *in vivo* was determined positive if both exhaled NO increased and systemic arterial blood pressure decreased (+). – denotes no NO donation capacity *in vivo*. UV absorbance in HPLC was denoted (+) if treatment with NO gas of parent compounds resulted in new chromatographic peaks exhibiting characteristic absorbance around 225 nm and vibrational absorbance in the range 330-370 nm (225). \*denotes that the new chromatographic peaks in HPLC had the same retention time and UV characteristics in HPLC as the corresponding authentic and commercially available organic nitrite. NO release in HPLC coupled to on-line nitrite reduction (LC-NO) was indicated (+) if new chromatographic peaks also were confirmed to release NO in the nitrite reduction system. <sup>#</sup> indicates that authentic organic nitrite of the parent compound also released NO at identical retention time in the LC-NO. Some of the formed compounds (+) were identified with gas chromatography-mass spectrometry (GC-MS). Non-filled spaces in the table indicate that the corresponding analysis was not performed for that compound in that system. Absence of markers <sup>#</sup> and \* means that commercial reference compounds were not available. Table 1 from paper III.

An important observation was that the lipid emulsion turned yellow indicating oxidation. In order to find a more pure NO donating solution and since the lipid emulsion contained glycerol, deoxygenated NO-treated glycerol (glycerol-NO; 25% in saline, v/v) was infused intravenously ( $100 \mu\text{l kg}^{-1} \text{min}^{-1}$ ) in another rabbit (pre-treated with L-NAME  $30 \text{ mg kg}^{-1}$ ) which resulted in increased FENO and decreased MAP. Exhaled NO and MAP remained unchanged during intravenous infusions of deoxygenated NO-treated saline and glycerol at the corresponding infusion rates. These experiments suggested that an NO donor solution was created when deoxygenated solutions of lipid emulsion or glycerol were treated with NO gas.

Thereafter more than 20 different deoxygenated NO-treated solutions of alcohols and carbohydrates (Table 1) were administered intravenously ( $100 \mu\text{l kg}^{-1} \text{min}^{-1}$ ) to rabbits in order to test their NO donating capabilities. The capacity of NO donation *in vivo* was determined positive if both FENO increased and MAP decreased. Deoxygenated NO-treated alcohols, polymers of alcohol, monosaccharides, monosaccharide alcohols, certain modified monosaccharides, disaccharides and carbohydrate polymers presented NO donating capacities (Table 1). Some of the NO donor solutions induced methaemoglobinaemia, e.g. propanol-NO (deoxygenated NO-treated propanol, 25% in saline). A common feature for the compounds exhibiting NO donating capacity was that they contained at least one hydroxyl group. Some compounds did not donate NO *in vivo* despite deoxygenation and NO gas treatment (Table 1), indicating that amine or carboxyl groups close to the hydroxyl-group in the substrate molecule prevented the formation of the NO donor compounds.

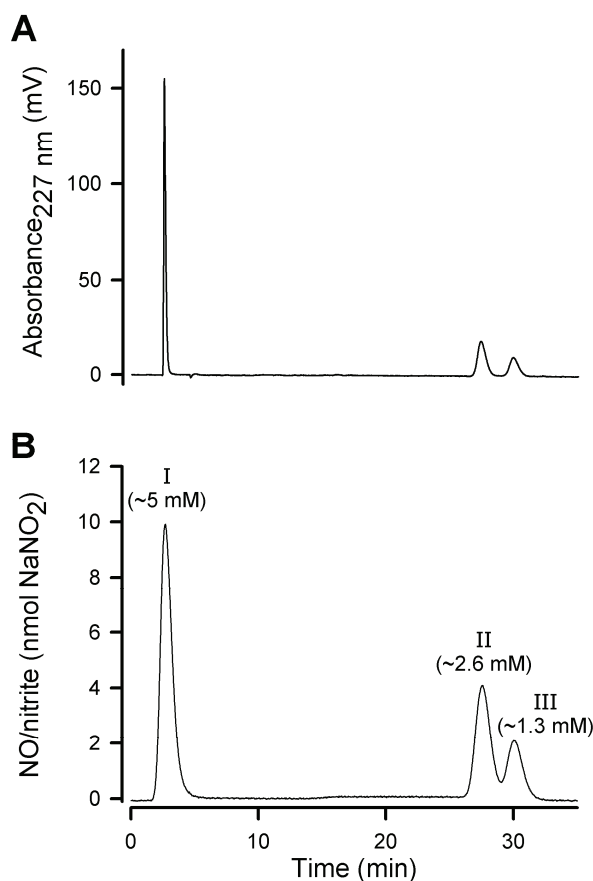
#### **4.4.2. Identification of reaction products with liquid chromatography coupled to on-line nitrite reduction (Paper III, IV)**

HPLC coupled to on-line nitrite reduction (LC-NO) was developed in order to further characterise and identify the NO donating compounds. In the chromatogram and in the synchronised NO/nitrite recording several peaks were identified. First, a peak which was regarded as emanating from the NO gas and inorganic nitrite appeared in the front (denoted peak I) of all the analysed NO solutions including deoxygenated NO-treated saline and water solutions of inorganic nitrite (Exemplified by analysis of PDNO in Figure 5). Furthermore in all the deoxygenated NO-treated solutions investigated with HPLC or LC-NO (Table 1) there also appeared UV absorbing peaks with UV absorbance maxima near 225 nm and with the, for organic nitrites characteristic, vibrational spectral peaks in the range 330-370 nm (225). All these novel chromatographic peaks were capable to generate NO in the nitrite reduction system (Table 1). Neither in the HPLC nor in LC-NO were these novel peaks seen when deoxygenated but non-NO treated organic starting materials were analysed. The number of eluted organic nitrite-like peaks correlated with the number of nitrosylation sites in the form of hydroxyls in the parent compounds: deoxygenated NO-treated propanol (propanol-NO) yielded one peak, deoxygenated NO-treated 1,2-propanediol (PDNO) exhibited two peaks (II and III in Figure 5) and deoxygenated NO-treated glycerol (glycerol-NO) gave two peaks and deoxygenated NO-treated glucose (glucose-NO) gave 4 organic nitrite-like peaks. These experiments suggested that the reaction products between NO gas and the organic starting material were organic mononitrites.

In these experiments the concentration of inorganic nitrite and organic nitrites in the NO donor solutions were determined, by comparison with aliquots of inorganic nitrite, to 1-5 mM in these experiments. Since the concentration of the organic starting material was 3.3 M the relation between reacted parent molecules to un-reacted parent molecules would be approximately 1:1000, if each reacted parent molecule bound one NO. Therefore the detection of the organic nitrites by nuclear magnetic resonance against this background was considered nearly futile. Attempts at identification with LC-MS yielded no conclusive data (unpublished results).

*Methodological considerations on HPLC coupled to on-line nitrite reduction*

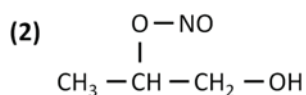
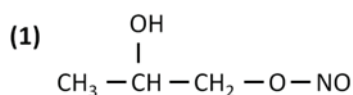
The present thesis introduced a method for analysing inorganic and organic nitrites in HPLC coupled to a nitrite reduction system. There are several methods for analysing nitrites (106). NO release from guinea-pig colon (103) and from rabbit corpus cavernosum (89) has previously been studied in our laboratory by using the nitrite reduction system alone. A partly similar system (isocratic HPLC elution coupled to nitrite reduction) to analyze inorganic nitrite in food, saliva and urine has been described (195). We used both isocratic and gradient elution in the HPLC and the method offered both high sensitivity (at least 10 pmoles, by the chemiluminescence) and specificity (different retention times by HPLC). The methods were used to identify organic nitrites that could not be identified with GC-MS or LC-MS. Caution must be taken in that some other compounds might give a signal in the ozone reaction (e.g. ethers) and that the nitrite reduction system can reduce both inorganic nitrite and organic nitrites, as well as N-nitrosylated and S-nitrosylated compounds (229).



**Figure 5.** HPLC (panel A) coupled to reflux NO/nitrites analysis (panel B) for identification of inorganic and organic nitrites. Chromatogram obtained at 227 nm (panel A) and nitrite content (panel B) of 2.5  $\mu$ l of deoxygenated NO-treated 1,2-propanediol solution (25% in saline, v/v), injected at time 0. Isocratic elution (10 mM ammonium formate in water) using a C<sub>18</sub> column. Peak I corresponded to NO/inorganic nitrite. Peak II and III had major UV absorbance maxima at 225 and 226 nm respectively and also exhibited vibrational spectra in the region 330-370 nm, characteristic for organic nitrites (225). Figure 4 in paper III.

#### 4.4.3. Identification of reaction products with gas chromatography-mass spectrometry (Paper III)

Using GC-MS, propyl nitrite was identified in the C<sub>18</sub> HPLC fractions corresponding to the novel chromatographic peak from propanol-NO. Gas chromatography-mass spectrometry of commercial propyl nitrite confirmed the identification, thus it was concluded that the reaction product in propanol-NO was propyl nitrite. Furthermore two novel compounds in the PDNO sample were identified by GC-MS. The mass spectra of these peaks were suggested to arise from the organic mononitrite of 1,2-propanediol when the hydroxyl at position 1 is nitrosylated (2-hydroxy propyl nitrite, compound (1) in Figure 6) and from the mononitrite of 1,2-propanediol when the hydroxyl at position 2 is nitrosylated (2-hydroxy-1-methylethyl nitrite, compound (2) in Figure 6). Mass spectrometry using chemical ionization showed the expected molecular ions in the mass spectra for both compound 1 and 2, thus verifying that the two reaction products in PDNO, also seen in LC-NO (Figure 6), were the two organic mononitrites of 1,2-propanediol. To our knowledge these compounds have never been identified before and they received CAS numbers based on our identification. Determination of the reaction products of glycerol-NO by the same method failed due to chromatographic interference from the large amounts of unreacted glycerol.



**Figure 6.** The two organic nitrites in the PDNO solution. **(1)** is 2-hydroxy propyl nitrite (IUPAC name: 1-(nitrosooxy)propan-2-ol; CAS no. 950478-72-5). **(2)** is 2-hydroxy-1-methylethyl nitrite (IUPAC name: 2-(nitrosooxy)propan-1-ol; CAS no. 950478-73-6). Figure 1 in paper IV.

#### 4.4.4. Comparisons with commercially available organic nitrites and the reaction products between NO gas and alcohol starting material (Paper III)

Further experiments were performed to verify the formation of organic nitrites. When analysing commercially available propyl nitrite and propyl nitrate there was a perfect match between the elution time and UV absorbance spectra between peak II in propanol-NO and authentic propyl nitrite, whereas the retention time and UV absorbance spectrum of propyl nitrate were completely different. The content of propyl nitrate, if any, in the propanol-NO must be less than 2.5% of the content of propyl nitrite (calculated on detection limits). Further comparisons with other commercial organic nitrites in HPLC and LC-NO were completely compatible with formation of the corresponding organic nitrites in the NO gas treated hydroxyl precursor solutions (Table 1). These experiments provided further evidence that the reaction products between NO gas and organic starting material yielded organic nitrites. Also interesting, in the experiments with authentic organic nitrites it was found that the stability of the authentic organic nitrites was poor in water and was markedly increased by dissolving them in deoxygenated water or more preferable in

deoxygenated NO gas-saturated solution. These experiments suggested that one advantage of our production method of organic nitrites was that deoxygenation and the presence of NO gas increased the stability of the organic nitrites in the solution.

#### **4.4.5. Summary of evidence that organic nitrites are formed in the reaction between NO gas and organic starting material containing at least one hydroxyl group (Paper III)**

The reaction products between propanol and NO gas and between 1,2-propanediol and NO gas were identified to be propyl nitrite, a previously known organic nitrite and the two possible organic mononitrites of 1,2-propanediol respectively (Figure 6). It is most likely that the other NO donor solutions also contain the organic mononitrites of the organic starting material, because: (1) They were produced with the same method that produced organic nitrites unequivocally identified by GC-MS; (2) They showed similar NO donor capabilities *in vivo*; (3) The number of reaction products was associated with the number of hydroxyl groups in the carrier molecule. (4) UV absorbance maxima were around 219-234 nm and they exhibited characteristic vibrational spectra (225); (5) They released NO in the nitrite reduction system. (6) The starting materials for the reactions were known.

The present thesis work did not aim to elucidate the mechanism by which the organic nitrites were formed in the reaction with NO gas. It is difficult to exclude trace amounts of oxygen in the reaction chamber. Therefore the reaction may involve a direct action of NO on the hydroxyl groups of the carrier molecules or it may involve a nitrosating agent such as N<sub>2</sub>O<sub>3</sub> (formed by the reaction of NO gas and trace amounts of oxygen) or it may be another, for us unknown, mechanism.

#### **4.4.6. Determination of the content of NO gas, inorganic nitrite and organic nitrites in the PDNO solution and deoxygenated NO-treated saline (Paper IV)**

Since the NO donor solutions contained NO gas and inorganic nitrite, which may also be bioactive (45), it was considered important to determine the content of NO-gas, inorganic and organic nitrite in the PDNO solution and in deoxygenated NO-treated saline (Saline+NO) and to perform dose-response experiments *in vivo* with appropriately selected solutions. In addition it was found that the content of organic nitrites could be increased by prolonging the time for purging the solution with NO gas and by using a small glass chamber. Therefore the contents of inorganic and organic nitrite are larger compared to that indicated in Figure 5, which is favourable in order to limit the infusion of un-reacted starting material and to make infusions in larger animals and humans more feasible. The NO gas content of the PDNO solution (0.6 mM) was determined to one third of the NO gas content in the Saline+NO solution (Table 2). The total contents of NO/nitrites in the PDNO solution were comparable to that in the Saline+NO solution (Table 2). The concentration of organic mononitrites of the PDNO solution was 15 times higher than that of NO gas in the same solution (Table 2).

Therefore in the dose-response experiments with PDNO (see below), a 1,2-propanediol solution (25% in saline, v/v) with addition of 20 mM of inorganic nitrite (PD+nitrite) and the deoxygenated NO-treated saline solution were infused as control solutions at

the same infusion rates as PDNO. Thereby the Saline+NO solution served as a control for both inorganic nitrite and NO gas (actually three times more NO gas). The PD+nitrite solution served as a control for the total inorganic nitrite content in combination with 1,2-propanediol.

**Table 2.** Determination of concentrations of nitrogen oxides in the PDNO solution (deoxygenated NO-treated 1,2-propanediol 25% v/v in isotonic saline) and its corresponding NO-saturated saline control, used in the dose-response experiments.

	<b>PDNO</b>	<b>Saline+NO</b>
<i>Direct injections in reflux system</i>		
<i>a)with nitrite reducing agent</i>		
Total NO gas + nitrites (mM)	20.0±0.2	22.0±0.4
<i>b)without nitrite reducing agent</i>		
NO gas (mM)	0.6±0.1	1.7±0.1
 <i>LC-NO (HPLC followed by nitrite reduction)</i>		
Inorganic nitrite=Peak I (mM)	7.8±0.1	20.2±0.5
Organic nitrites=Peak II+III (mM)	9.1±0.1	0
 <i>Deduced total content of nitrogen oxides</i>		
Peak I+II+III + NO gas (mM)	17.5±0.1	21.9±0.6

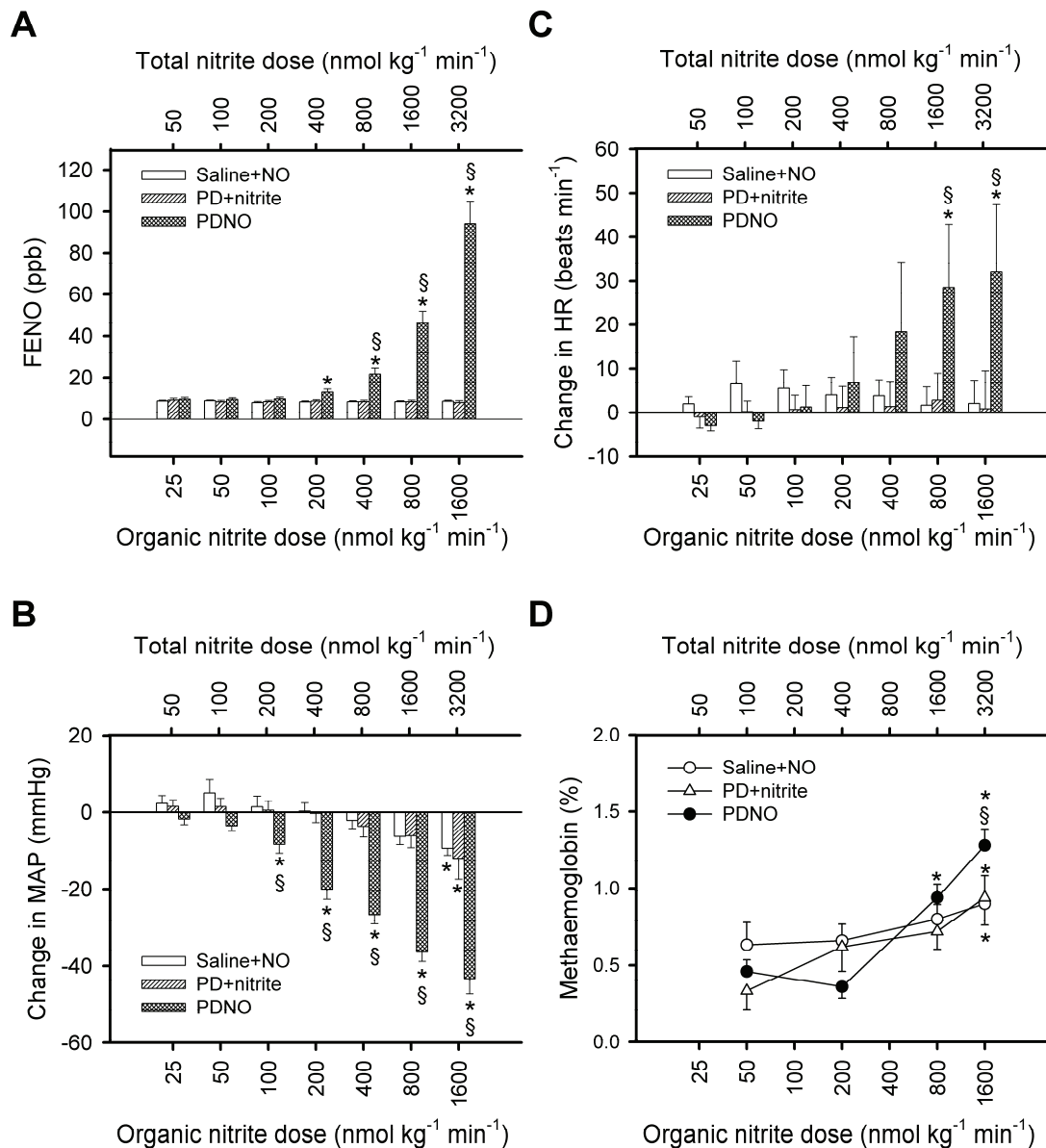
Samples were analysed by means of direct injections into the reflux system for nitrite reduction coupled to chemiluminescence detection (total NO gas/nitrite determination) and without nitrite reducing agent (NO gas determination) and by means of LC-NO, i.e. HPLC (isocratic C<sub>18</sub> column, elution with 7% of acetonitrile in 10 mM ammonium formate) followed by on-line nitrite reduction in the reflux system (peak I, II, III in Figure 5). N=3-6. Peak I in the HPLC eluted at the retention time of inorganic nitrite and was deemed free of NO gas as judged from experiments in the absence of reducing agent (data not shown). Peak II and III had previously been shown to be the organic nitrites in Figure 6. Table 1 in paper IV.

#### 4.4.7. Further exploration of *in vivo* effects of new organic nitrites and in comparison with NO gas and inorganic nitrite (Paper III, IV)

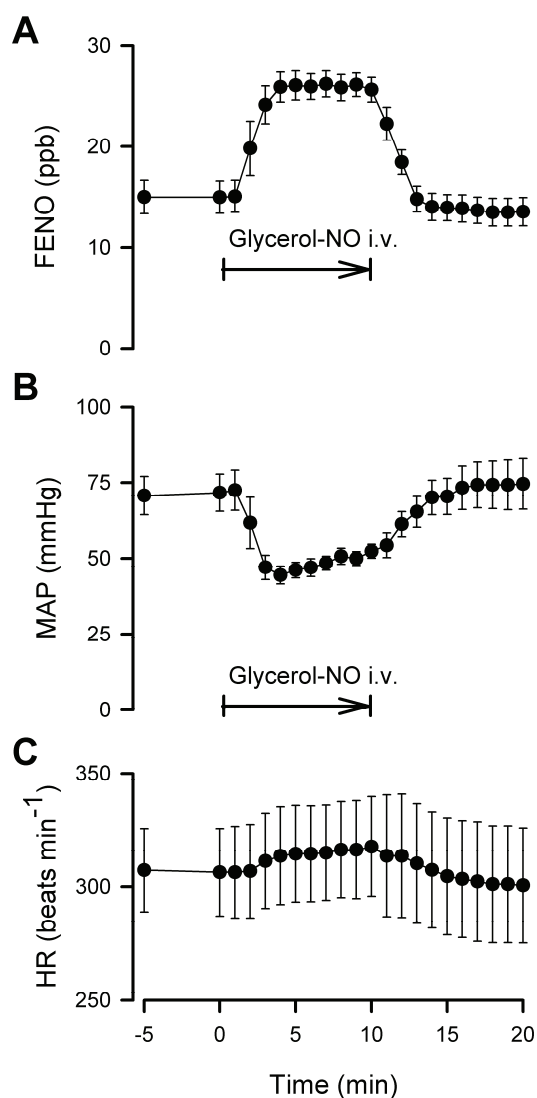
PDNO increased FENO and decreased MAP dose-dependently (Figure 7), whereas Saline+NO and PD+nitrite, at the highest doses used, only had a small effect on MAP without affecting FENO (Figure 7). Methaemoglobin increased at the highest doses of all three solutions but remained below 2.0% in all animals (Figure 7). These experiments clearly show that PDNO is a powerful vasodilator, even in the systemic circulation, at the highest doses. Compared with inorganic nitrite at similar doses, PDNO is more than 15 times more potent on MAP. These data also suggest that it is the inorganic nitrite and not the NO gas of the Saline+NO solution which is bioactive. Importantly, hemodynamic effects of inorganic nitrite, in contrast to PDNO, are associated with methaemoglobin. This is not surprising since the present theory for activation of inorganic nitrite to bioactive NO by deoxyhaemoglobin involves formation of methaemoglobin (45). Also in humans, systemic hemodynamic effects of inorganic nitrite are associated with severe methaemoglobin formation (45, 97). In dogs, inorganic nitrite ( $450 \text{ nmol kg}^{-1} \text{ min}^{-1}$  for 15 min followed by  $280 \text{ nmol kg}^{-1} \text{ min}^{-1}$  for 105 min) decreased systemic blood pressure (by approximately 30 mmHg) and also decreased cardiac output, but unfortunately methaemoglobin was not monitored (57). These findings indicate that dogs and humans are more sensitive to inorganic nitrite than rabbits. It may also be that other species compared to rabbits are more sensitive to PDNO. Experiments with piglets and sheep indicate that those species are at least 4 times more sensitive to PDNO and that bioactive doses of PDNO are  $30\text{-}60 \text{ nmol kg}^{-1} \text{ min}^{-1}$  in sheep and that corresponding control infusions of inorganic nitrite ( $60\text{-}120 \text{ nmol kg}^{-1} \text{ min}^{-1}$ ) are inactive (unpublished data).

Furthermore, during infusions of the organic nitrite solutions both FENO and MAP quickly reached a stable plateau (exemplified by Glycerol-NO in Figure 8), indicating absence of acute tachyphylaxis. This is in contrast to nitroglycerin where a peak and plateau pattern has been observed and was taken as an indication of acute tachyphylaxis (13). The lack of acute tolerance in other organic nitrites has previously been shown both *in vivo* and *in vitro* (23). This important difference and perhaps other differences between organic nitrites and organic nitrates could encourage the use of organic nitrites instead of organic nitrates in clinical settings.





**Figure 7.** Dose-response studies of nitrites in anaesthetised and ventilated rabbits. Exhaled NO (FENO, panel A), change in systemic arterial blood pressure (MAP, panel B), change in heart rate (HR, panel C) and methaemoglobin (panel D) in ventilated rabbits subjected to infusion of PDNO (organic nitrite), NO gas treated saline (Saline+NO) and inorganic nitrite (20 mM) in 1,2-propanediol (25% in saline, v/v) at increasing dose rates. The lower and upper x-axis in each panel show the dose of organic nitrite (for the PDNO) and the total dose of nitrites (organic nitrites + inorganic nitrite) of all the infusions, respectively. \* denotes a significant difference compared to baseline within that group. § denotes a significant difference between the PDNO group compared to both PD+nitrite and Saline+NO groups for that particular dose. N=4-5 for each infusion. Figure 3 in paper IV.

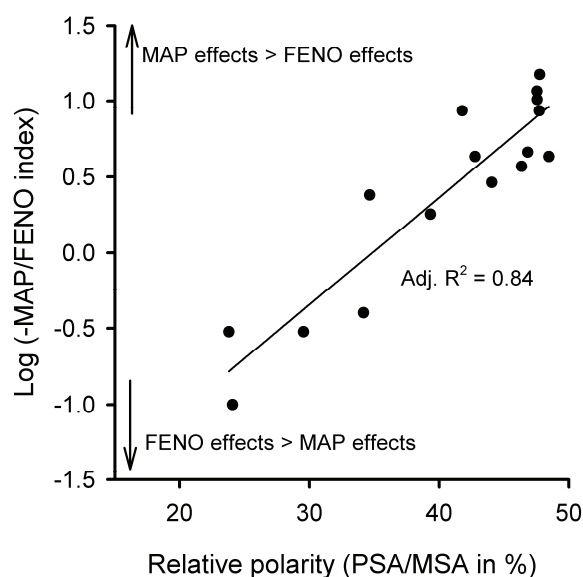


**Figure 8.** Anaesthetised and ventilated rabbits ( $n=5$ ). Exhaled NO (FENO, panel A), mean arterial blood pressure (MAP, panel B) and heart rate (HR, panel C) during an intravenous infusion ( $30 \text{ ul kg}^{-1} \text{ min}^{-1}$ , for 10 min) of glycerol-NO (25% glycerol in saline, v/v, treated with NO gas and identified by LC-NO to contain glyceryl nitrites). Note the plateau pattern of the response, indication no acute tolerance development. Figure 3 in paper III.

#### 4.4.8. Determination of molecular physico-chemical properties of the organic nitrites that are important for selectivity for the pulmonary and systemic circulation respectively (Paper III)

In the present work it was early noted that some organic nitrite solutions had large effects on FENO and minor effects on MAP. On the other hand other organic nitrites had large effects on MAP and small effects on FENO. A previously published MAP/FENO-index was used (13) that described these differential effects on MAP versus FENO, exerted by the different organic nitrites and taken as a marker for selectivity towards the systemic versus pulmonary circulation (refer to paper III for calculations). The interpretation was that a MAP/FENO-index away from zero indicated increased relative selectivity towards the systemic circulation and a MAP/FENO-index closer to zero denoted a relative selectivity towards the pulmonary circulation. It was hypothesised that the selectivity of the individual organic nitrite depended on properties of that molecule. It was found by using multiple regression that the relative surface polarity of the organic nitrite molecule was the best predictor

of the selectivity, i.e. the more relative surface polarity of the NO donor molecule, the more effects on MAP compared to FENO and vice versa (Figure 9). The most non-polar organic nitrites induced methaemoglobin. Thereafter, PDNO and glycerol-NO were chosen as candidates to proceed with in further experimentation since they exhibited increased selectivity towards the pulmonary circulation, negligible methaemoglobin formation and low toxicity. Note that the relative selectivity may be overridden by a high dose of e.g. PDNO and glycerol-NO (Figure 7 and 8). Glucose-NO was chosen as a candidate for comparison with increased selectivity towards the systemic circulation.



**Figure 9.** Anaesthetised and ventilated rabbits subjected to intravenous infusions of different organic nitrite NO donor solutions. The MAP/FENO indices were calculated from experiments with intravenous infusions of NO donor solutions by dividing the change in mean systemic arterial blood pressure (MAP, % of baseline) with increase in exhaled NO (FENO, delta ppb compared with baseline). Each point represents one NO donor (deoxygenated and NO-treated 1-Propanol – Lactobionic acid in Table 1). Shown is also the best linear regression found by exploring the dependent variable Log (-MAP/FENO index) against the independent variable relative polarity (polar surface area divided with molecular surface area in %, PSA/MSA) for the different NO donors showing that relative polarity is a strong predictor for MAP versus FENO effects. Figure 5 in paper III.

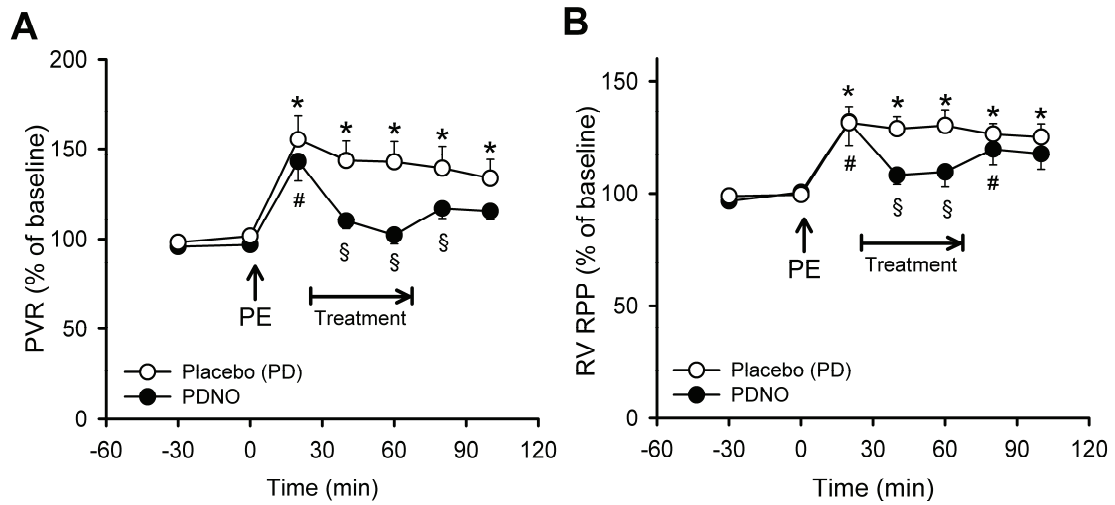
#### 4.4.9. Effects of certain organic nitrites in pharmacologically induced pulmonary hypertension (Paper III)

The three candidates (PDNO, glycerol-NO and glucose-NO) were tested in a model of pharmacologically induced pulmonary hypertension (U46619, 150-300 ng kg<sup>-1</sup> min<sup>-1</sup>) in a few rabbits. PDNO and glycerol-NO given intravenously effectively reversed the induced pulmonary hypertension with minor effects on MAP, unchanged blood gases and no methaemoglobin formation. Interestingly, glucose-NO also partially lowered the pulmonary hypertension but this effect was paralleled with severe systemic

hypotension. Thereby these experiments suggested, although not completely proved, that the differential effects on FENO and MAP of organic nitrites intravenously may be used as an indication of selectivity towards the pulmonary versus the systemic circulation. The MAP/FENO-index suggested that PDNO was slightly more selective for the pulmonary circulation compared with glycerol-NO and therefore PDNO was further investigated in acute pulmonary embolism.

#### **4.5. EFFECTS OF PDNO IN ACUTE EXPERIMENTAL PULMONARY EMBOLISM (PAPER IV)**

In two groups of rabbits where the pulmonary circulation and gas exchange parameters were monitored acute pulmonary embolisation (30 mg kg<sup>-1</sup> homogenised muscle) resulted in the expected changes of the measured parameters (see above), including pulmonary hypertension (Figure 10), increased right ventricle rate-pressure product (Figure 10), increased physiological dead-space ventilation, and increased venous admixture. Treatment with either PDNO (200 nmol kg<sup>-1</sup> min<sup>-1</sup>) or 1,2-propanediol (PD; 25% in saline, v/v, at the same infusion rate) was performed 20-60 min after pulmonary embolisation. PDNO increased FENO, decreased and normalised pulmonary vascular resistance and right ventricle rate-pressure product (Figure 10) whereas PD did not have these effects. PDNO decreased pulmonary arterial pressure, compared to values before start of treatment. The effects of PDNO were reversed when stopping the treatment (Figure 10). Systemic arterial blood pressure was similar in both groups. PDNO and PD did not change other measured parameters. Methaemoglobin was very modestly increased (by approximately 0.3%) at the end of PDNO treatment, compared to PD and baseline, and importantly all animals had methaemoglobin <1% during the study period.



**Figure 10.** Anaesthetised and ventilated rabbits. The response of pulmonary vascular resistance (PVR, panel A) and right ventricle rate pressure products (RV RPP, panel B) to pulmonary embolism (PE) induced at time 0 min and to treatment started at 20 min. The two groups received placebo (1,2-propanediol, PD) or 200 nmol kg<sup>-1</sup> min<sup>-1</sup> of 1,2-propanediol mononitrites (PDNO). \* and # denote a significant difference compared to baseline in the placebo group and in the PDNO group, respectively. § denotes a significant difference between the two groups at the actual time point. n=6 in each group. Part of Figure 5 in paper IV.

## 5. GENERAL DISCUSSION

One major finding in the present thesis is that exhaled NO increases during acute experimental pulmonary embolism. Another major finding is that endogenous NO is protective in acute experimental pulmonary embolism. A third major finding is that treatment with NO donating organic nitrites intravenously in acute pulmonary embolism is beneficial. These findings provide further knowledge on the function of NO in the lung and in particular in acute pulmonary embolism and certain aspects of these findings will be discussed below. In addition exhaled NO emerges as a method that should be tested in humans suffering from acute pulmonary embolism. Furthermore new organic nitrites were discovered and models describing their selectivity towards the pulmonary versus systemic circulation were developed. The mechanisms behind this selectivity will be discussed below as well as potential future use of these or other new organic nitrites in diseases with compromised pulmonary circulation.

### **5.1. MECHANISMS BEHIND THE INCREASE IN EXHALED NO IN ACUTE PULMONARY EMBOLISM**

Several mechanisms that involve increased production and/or decreased elimination of pulmonary NO might contribute to the increase in exhaled NO following PE:

#### **5.1.1. Regulation of pulmonary NO production by airway/alveolar CO<sub>2</sub> and O<sub>2</sub>**

Acute pulmonary embolism leads to un-perfused or partly perfused parts in the lung. In these lung parts the alveolar content of CO<sub>2</sub> is low (detected as decreased end-tidal CO<sub>2</sub>, paper I, II, IV) and the alveolar content of O<sub>2</sub> is increased (detected as increased end-tidal O<sub>2</sub>, data not shown). As outlined in the introduction these two changes and especially the decrease in CO<sub>2</sub> concentration will increase the pulmonary NO production and thus increase exhaled NO (4, 210, 243). Indeed, the present work shows that approximately 50% of the increase in exhaled NO after acute pulmonary embolism could be explained by the decreased alveolar CO<sub>2</sub>.

#### **5.1.2. Release of NO synthase stimulating substances**

Acute pulmonary embolisation is known to lead to the release of bioactive substances as outlined in the introduction (200, 209), which potentially can stimulate NO production and thus increase exhaled NO (209).

##### *Endothelin*

Endothelin-1 infusion has been shown to increase exhaled NO (134) and a non-selective endothelin receptor antagonist decreased exhaled NO (95), thus indicating that endothelin-1 released during acute pulmonary embolism has the potential to increase exhaled NO. It has been proposed that the increase in exhaled NO due to endothelin-1 is mediated by activation of the endothelin-A-receptor (75).

### *Serotonin*

Treatment for 30 days of guinea-pigs with a serotonin re-uptake inhibitor increased exhaled NO, but lung inflammation occurred making interpretation difficult (32). It has been suggested that the pulmonary vasoconstrictive response to serotonin is partly attenuated by pulmonary endothelial NO release (144), but whether this tentative release can be seen as change in exhaled NO is uncertain.

### *Thromboxane A<sub>2</sub>*

It is not likely that release of thromboxane A<sub>2</sub> increases exhaled NO since intravenous infusion of thromboxane A<sub>2</sub>-mimetic U46619 in the present work and previous studies does not change exhaled NO (46, 85).

#### **5.1.3. Reduced scavenging of NO by haemoglobin**

Reduced binding of pulmonary NO to haemoglobin in the erythrocytes, owing to reduced blood flow and diminished blood volume in the lung capillaries, may increase exhaled NO (26). In support of this, completely stopping the pulmonary blood flow has been shown to increase exhaled NO (88). Furthermore it has been shown that occlusion of pulmonary arteries by wedging a pulmonary arterial catheter rapidly increases exhaled NO from the lung regions with occluded vessels (71). The increase in exhaled NO correlated with the decrease in blood flow but not with the increase in pulmonary arterial pressure (71).

#### **5.1.4. Regulation of inducible NO synthase**

Up-regulating the iNOS-gene, as in asthma, might be of importance in a later phase of pulmonary embolism. However, iNOS is not likely to be important in the acute phase owing to the time lag in induction of the enzyme (56).

#### **5.1.5. Effects of changes in blood gases**

Despite profound changes in blood gases during pulmonary embolism, the lowered arterial partial pressure of O<sub>2</sub> (11, 100), the increased arterial partial pressure of CO<sub>2</sub> (243) and the decreased arterial pH (4) are not within the ranges needed to alter exhaled NO. From experiments with isolated buffer-perfused lungs, it has been convincingly shown that it is the airway/alveolar, and not the intravascular, concentration of CO<sub>2</sub> and O<sub>2</sub> that regulates exhaled NO (100, 243).

#### **5.1.6. The role of NO in matching ventilation and perfusion in the lung**

It is an intriguing idea that the increase in exhaled NO after pulmonary embolisation may be a sign of mechanisms in the lung geared towards increasing perfusion to well-ventilated parts that are completely or partly obstructed by emboli or constricted due to the release of mediators. Well-ventilated and inadequately perfused lung parts are stretched and are characterised by low CO<sub>2</sub> and high O<sub>2</sub> concentrations and attenuated scavenging of NO by haemoglobin which all increase pulmonary NO production, as outlined above and in the introduction. Thus it has been suggested that the increased local NO concentration, in well-ventilated lung parts, may dilate the associated pulmonary vessel and thereby matches local ventilation and perfusion (6). Future studies are needed to further confirm this idea and these studies should preferably be

done in humans or in animals where the function and regulation of pulmonary NO is similar to humans.

## **5.2. POTENTIAL USE OF EXHALED NO IN ACUTE PULMONARY EMBOLISM**

The present thesis work (paper I, II, IV) indicates that measurement of exhaled NO has a potential of being a marker for pulmonary embolisation of different genesis especially in the very acute phase. Of course this issue needs to be carefully evaluated in clinical studies involving humans suffering from acute pulmonary embolisation, but if it works it would be useful. Problems that need to be overcome include the interindividual variation of levels of NO in exhaled breath, establishing cut-off values and how to differentiate between other pulmonary diseases, in particular those associated with pulmonary inflammation, e.g. asthma (21). Another inherent problem is that the increase in exhaled NO one hour after acute pulmonary embolisation was small. On the other hand, it might not necessarily be so in humans.

A situation where exhaled NO may be used, especially since the present study (paper II) shows that the increase in exhaled NO is dependent on emboli dose, i.e. severity, is when determining on the need for more intense treatment (e.g. thrombolysis), instead of only anticoagulation. It is known that although hemodynamically stable approximately 50% of the patients suffering from acute pulmonary embolism will show signs of right ventricular dysfunction, which is a negative prognostic predictor (82, 124). Therefore a subgroup of these patients is believed to benefit from more intense treatment (e.g. thrombolysis) but how to identify these patients remains an open question (125). Most of the nowadays used markers for risk stratification include measures of right ventricular dysfunction (echocardiographical signs or biochemical markers) (124) and maybe a combination of these will not improve the risk stratification that much due to redundancy. Instead a combination of a marker for right ventricle dysfunction and a marker for the affection of pulmonary embolisation on pulmonary function (e.g. exhaled NO) may improve risk stratification in the future.

## **5.3. PROTECTIVE MECHANISMS OF ENDOGENOUS AND EXOGENOUS NO IN ACUTE PULMONARY EMBOLISM**

The present studies clearly show that endogenous NO protects against the detrimental effects of acute pulmonary embolism and that inhibition of the endogenous NO production in combination with acute pulmonary embolism severely reduces survival (paper I, II). Furthermore, the present work shows that exogenous NO has beneficial effects in acute pulmonary embolism (paper IV). Due to the widespread and diverse functions of NO in the body including the lung it may be hard to suggest one protective mechanism of endogenous and exogenous NO in acute pulmonary embolism. Probably it is a combination of the following:

### **5.3.1. Counteraction of pulmonary hypertension**

As outlined in the introduction endogenous NO formation modulates and counteracts pulmonary hypertension induced by hypoxia and a thromboxane A<sub>2</sub> mimetic in



rabbits (177, 228), by serotonin in cats (144) and by endothelin-1 in rats and in piglets (39, 134). All these three vasoconstrictors classes have been shown to be released in acute pulmonary embolism (201) and therefore it is likely that one protective mechanism of endogenous NO is counteraction of the pulmonary hypertension of acute pulmonary embolism. Likewise, exogenous NO, e.g. organic nitrites, will act by vasodilating the pulmonary circulation, thus counteracting the increased pulmonary vascular resistance due to the released vasoactive mediators and endothelial dysfunction (seen as attenuated response to acetylcholine) in the pulmonary vasculature (72, 201). Indeed, in the present work it is shown that PDNO and glycerol-NO are effective pulmonary vasodilators in thromboxane A<sub>2</sub>-induced pulmonary hypertension (paper III) and that PDNO effectively decreases pulmonary vascular resistance in acute pulmonary embolism (paper IV). In addition, PDNO produced vasodilation of the pulmonary circulation that led to decreased work load on the right heart and thus decreased oxygen consumption by the right heart (shown by decreased rate-pressure product, paper IV), which is beneficial in a condition associated with hypoxaemia.

### **5.3.2. Modulation of perfusion and contractility of the right ventricle**

In humans and rabbits endogenous NO is a modulator of systemic vascular resistance (206) and the perfusion of vascular beds, e.g. skeletal muscle (176). Importantly endogenous NO is a regulator of coronary blood flow to the right ventricle at rest, in global hypoxia (and thus hypoxemia) and in pulmonary hypertension (136, 247). Acute pulmonary embolism includes hypoxemia and pulmonary hypertension; therefore one protective mechanism of endogenous NO which might be by regulation of coronary perfusion in this condition. It is also known that endogenous NO is a regulator of heart performance (171) and that acute NOS inhibition reduces myocardial oxygen consumption and myocardial contractility (198). It has been shown that a low dose of NO donors increased the contractility of isolated rat myocytes whereas a high dose of NO donors decreased the contractile response (118). However the actual role of NO in myocardial performance is complex and is debated due to conflicting results (171) and it is hard to predict the effect of exogenous NO on contractility. If treatment with PDNO will further increase blood flow to the right ventricle in a healthy laboratory animal is uncertain, but we speculate that increased oxygen supply to the right ventricle may be another beneficial effect of PDNO in acute pulmonary embolism. This effect may be important in a human suffering from acute pulmonary embolism with risk factors for atherosclerosis and endothelial dysfunction where the endothelial NO production is diminished (184, 185). One important finding together with this is that the treatment did not result in decreased systemic blood pressure, since systemic arterial blood pressure is an important determinant of perfusion of the right myocardium especially in pulmonary hypertension (237).

### **5.3.3. Modulation of platelet aggregation**

Endogenous platelet-derived NO modulates platelet aggregation and inhibition of endogenous NO synthesis leads to enhanced aggregation of thrombocytes (187). It has been shown that inhibited NO production augments platelet aggregation *in vivo*

induced by adenosine diphosphate, platelet activating factor or thrombin (138). It is postulated that endothelial-derived NO inhibits platelet adhesion and aggregation whereas platelet-derived NO limits further recruitment of thrombocytes, modulates granule secretion, including vasoconstrictive substances such as serotonin and ADP, and restricts thrombus growth (155, 162). Furthermore endogenous NO may inhibit platelet aggregation by inhibiting exocytosis of the endothelial cells (137). Since it is known that platelets are activated by pulmonary emboli (40, 223), endogenous NO in pulmonary embolism may limit further aggregation and recruitment to the emboli and maybe more importantly result in decreased release of vasoconstrictive substances from the thrombocytes. Indeed, Emerson *et al.* (69) have previously shown that NO-synthase inhibition reduces the dose of collagen+adrenalin required to induce thromboembolic mortality. NO donors and authentic NO inhibit platelet aggregation (145, 242), which may inhibit thrombin-induced  $\text{Ca}^{2+}$  mobilisation and the subsequent release of vasoconstrictive agents (e.g. serotonin) from activated platelets (109) thus reducing pulmonary hypertension. Therefore in addition to a direct vasodilating effect of PDNO in acute pulmonary embolism (paper IV), it may also decrease the pulmonary hypertension by inhibiting the release of vasoconstrictors.

#### **5.4. POTENTIAL USE OF DRUGS AFFECTING THE NO SYSTEM IN ACUTE PULMONARY EMBOLISM**

The present thesis work introduces the use of organic nitrites in acute pulmonary embolism. PDNO showed significantly beneficial effects in this study by decreasing and normalising the pulmonary vascular resistance (by almost 100%) and thereby decreasing the work for the right ventricle (paper IV). The mechanisms behind these effects are discussed above. No severe adverse effects were noted; systemic arterial blood pressure was maintained due to the relative selectivity towards the pulmonary circulation, the shunt fraction was unchanged, methaemoglobin remained low and tolerance did not develop.

Several studies have examined the effects of supplying NO or of enhancing the effects of endogenous NO or a combination thereof, in acute pulmonary embolism and the major part of them are described in the introduction. Intravenous nitroglycerin and sodium nitroprusside have some beneficial effects on the pulmonary hypertension in acute pulmonary embolism, however their use in this condition is severely hampered by the systemic hypotension they induce (143). Previous studies showed that inhaled NO after acute pulmonary embolisation attenuates part (up to 40%) of the increase in pulmonary vascular resistance without systemic hypotension; however the effects on pulmonary gas exchange were conflicting (219). Since the initiation of this thesis project, it has been shown in a canine model of pulmonary microembolism that the NO donor diethylenetriamine/nonoate did not produce pulmonary vasodilation without development of severe systemic hypotension (61). Inorganic nitrite (450 nmol  $\text{kg}^{-1} \text{min}^{-1}$  for 15 min followed by 280 nmol  $\text{kg}^{-1} \text{min}^{-1}$  for 105 min) showed some beneficial effects of the pulmonary hypertension, by increasing cardiac output with no effects on the pulmonary arterial pressure, in dogs embolised with autologous blood clots (57). In pulmonary microembolism in dogs sildenafil attenuated approximately

50% of the increase in pulmonary vascular resistance without affecting the systemic arterial blood pressure (62). In another study by the same group, the beneficial effects of inorganic nitrite were enhanced by combined treatment with sildenafil, however systemic arterial blood pressure also decreased (58). These studies suggest that inorganic nitrite is beneficial in acute pulmonary embolism but the present data (paper IV) show that doses of inorganic nitrite that have hemodynamic effects also induce methaemoglobin, which unfortunately was not measured in the study by Dias-Junior *et al.* (58). In contrast, the dose of PDNO used in the present study (paper IV) only resulted in an increase in methaemoglobin by 0.3%, and future studies investigating the effects of inorganic nitrite should preferably include measurements of methaemoglobin. The present work indicates that intravenous treatment with PDNO produces larger effects on pulmonary vascular resistance than inhaled NO, demonstrating that intravenous administration of lung selective organic nitrites may reach larger vessels.

#### *Indications for NO donor treatment in acute pulmonary embolism*

Taken together, several studies including the present thesis work (paper IV) show beneficial effects of exogenous NO in acute pulmonary embolism. Since the majority of the possibly preventable deaths (i.e. diagnosed) in acute pulmonary embolism (241) occur in the first few hours, one indication of treatment with organic nitrites could be as a life-saving treatment in hemodynamically unstable patients where it is known that the mortality is very high (25-50%) (82). Another possible indication would be in hemodynamically stable patients with right ventricular dysfunction where the mortality is significantly higher than in patients without right ventricular dysfunction (124). The beneficial effects of thrombolysis on systemic and pulmonary hemodynamics as well as right ventricular function are significant the first days, whereas one week after the pulmonary embolisation, thrombolytic treatment and anticoagulation treatment have resulted in a similar improvement (120). Since thrombolysis is associated with risk of major bleeding, it would be beneficial for these patients to be treated with anticoagulation if the right ventricular function can be preserved by the use of a pulmonary vasodilator.

### **5.5. MECHANISMS OF SELECTIVITY AND LOW TENDENCY FOR METHAEMGLOBIN FORMATION OF ORGANIC NITRITES**

In the present study it was found that the relative selectivity towards the pulmonary and systemic circulation respectively of different organic nitrites varied (paper III). By use of calculated physico-chemical descriptors of the organic nitrites it was found that the relative polarity of the organic nitrite molecule determined the selectivity, i.e. the less relative polarity of the molecule the more effects in the pulmonary circulation versus the systemic circulation. Furthermore the most non-polar organic nitrites induced methaemoglobin formation. Relative polarity can be regarded as a measure of lipophilicity. It is known that several measures of compound lipophilicity correlate well with cell membrane passage (123). Therefore it may be speculated that very non-polar organic nitrite compounds very easily passed through the cell membrane of the red blood cell and therefore came in close contact with haemoglobin, directly reacted

with haemoglobin or released the NO molecule that immediately reacted with the haemoglobin, and produced methaemoglobin (64, 130). Less non-polar compounds did not penetrate the cell membrane of the red blood cells that easily and therefore did not produce methaemoglobin. Interestingly the half-life of free NO in blood is estimated to 2 ms, due to the rapid reaction with haemoglobin to form methemoglobin, and the rate of this reaction is solely diffusion-limited (130). This explains why NO gas dissolved in saline did not have hemodynamic effects other than those that were attributed to inorganic nitrite in the solution and it also shows that NO in close contact with haemoglobin will produce methaemoglobin. Therefore the permeability through the cell membrane of the red blood cells may determine tendency to methaemoglobin formation. The same reasoning might be applied to the endothelial cell layer in the pulmonary circulation, i.e. less polar compounds penetrate the endothelial cell barrier more easily and reach the smooth muscle cells in the pulmonary arteries and also more easily release NO which is then detected in exhaled gas. More polar compounds are not cleared that much in the pulmonary circulation and a higher concentration of the compound reaches the systemic circulation and therefore produces more effects on systemic arterial blood pressure. This idea agrees with the finding that higher doses of PDNO led to more active compound reaching the systemic circulation and thereby produced systemic hypotensive effects. It is important to emphasise that these high doses were not needed to affect the pulmonary hypertension. Mechanisms which have been shown to differ between organic nitrites are differences in efficacy in methaemoglobin formation (64), rate of hydrolysis (65), rate of transnitrosylation (65) and rate of enzymatic bioactivation the lung (147). Also the half-lives of some organic nitrites are very short. In a study of intravenous infusions of isobutyl nitrite in rats, the half-life, systematic clearance and distribution volume of this organic nitrite were determined to 1.3 min with a monoexponential decline, 10 times larger than cardiac output and  $5.8 \text{ l kg}^{-1}$  respectively (115). An issue that relates to the potential adverse effects of organic nitrites and especially in our study where there is a surplus of un-reacted alcohol and sugar is the toxicity of the carrier media itself. The toxicity of 1,2-propanediol has been extensively studied as it is used in pharmaceutical intravenous compositions of for example vitamins and benzodiazepines (36, 245). The toxicity is considered very low with no mutagenic activity and a dose of  $1 \text{ g kg}^{-1} \text{ day}^{-1}$  is considered safe in humans (36, 245). Therefore due to the beneficial effects of PDNO in acute pulmonary embolism and due to the absence of adverse effects including toxicity, systemic hypotension and methaemoglobin formation, a continued development of PDNO in order to reach clinical use in pulmonary hypertension could be considered.

## **5.6. FUTURE USE OF ORGANIC NITRITES IN DISEASES WITH COMPROMISED PULMONARY CIRCULATION**

The effects of these new organic nitrites, especially PDNO, should be investigated in other experimental models of diseases where the pulmonary circulation is compromised. One such model is ischemia-reperfusion of the lower body after aortic clamping, thus mimicking abdominal aorta aneurysm surgery. Abdominal aorta aneurysm is quite common in the general population and is life-threatening if

ruptured. Screening for abdominal aorta aneurysm reduces the mortality even though the post-operative mortality after elective surgery may be as high as 3% (83). The surgery includes aortic cross-clamping and subsequent reperfusion, which can cause multiple-organ failure including the lung in up to 25% of the cases (80). Several of the mechanisms for the lung injury are similar to acute pulmonary embolism: pulmonary microembolisation and the release of pro-inflammatory vasoactive mediators (79). Therefore treatment with organic nitrites, e.g. PDNO, could be life-saving by counteracting the lung injury and perhaps also the multiple-organ failure.

## **6. GENERAL CONCLUSIONS**

1. Models of acute pulmonary embolism and for simultaneous investigation of endogenous and exogenous NO have been developed in the rabbit, an easily available and manageable laboratory animal.
2. HPLC coupled to on-line nitrite reduction has been developed and is a sensitive method for identifying and quantifying organic and inorganic nitrites. Future use of this method may include analyses of body fluids in search for endogenous organic nitrites.
3. Exhaled NO is increased in acute pulmonary embolism of different genesis, and part of this increase is explained by the disturbed ventilation/perfusion and probably also by mediator release.
4. The endogenous NO system is protective both on hemodynamic parameters and on survival in acute pulmonary embolism of different genesis, further strengthening the idea that NO is a fundamental regulator of the pulmonary and cardiovascular systems.
5. Organic nitrites ready for intravenous infusion can be produced by the reaction of NO gas with some compounds in aqueous solutions, containing a hydroxyl-group not in vicinity of amine or carboxyl groups.
6. Organic nitrites, although one chemical entity in the sense of the O-N=O bond, is not a singular pharmacological entity. The selectivity towards the pulmonary and systemic circulation of organic nitrites is dependent on the relative polarity of the molecule. Methaemoglobin formation is a serious adverse effect of some organic nitrites whereas certain organic nitrites do not induce methaemoglobinemia.
7. Certain organic nitrites administered intravenously have beneficial effects in acute pulmonary embolism by counteracting the pulmonary hypertension, and thus decrease the oxygen demand of the right heart without significant adverse effects such as systemic hypotension, worsening of arterial oxygenation, methemoglobin formation or tolerance development.
8. Organic nitrites are more than 15 times more potent on a molar basis than inorganic nitrite in respect to hemodynamic effects, and those organic nitrites which exhibit little or no methemoglobin formation are potential therapeutics in situations with compromised pulmonary circulation, such as in pulmonary embolism and in multiple-organ failure.

## **7. POPULÄRVETENSKAPLIG SAMMANFATTNING**

Lungembolism, d.v.s. blodproppar i lungans blodkärl, är en vanlig sjukdom i befolkningen, med hög dödlighet (ca 15%). Sjukdomen beror vanligtvis på att en eller flera blodproppar som har bildats i benens och bäckenets vener plötsligt lossnar och förs med blodflödet via hjärtat till lungcirkulationen (lilla kretsloppet) där de fastnar. En plötslig blodtrycksstegring i lungcirkulationen uppstår, vilket är påfrestande för högra hjärthalvan. Lungans gasutbytesfunktion försämras samtidigt och det uppstår syrebrist i blodet, vilket påverkar hjärtat då det behöver syrgas för att utföra sitt arbete. Högerhjärtat kan börja svikta vilket får en allvarlig påverkan på hela kroppens blodcirkulation. Tillståndet är livshotande och läkemedelsbehandling för att lösa upp blodproppen bör snarast påbörjas. Att sänka blodtrycket i lungcirkulationen med en kärlvidgande behandling för att underlätta högerhjärtats arbetsbörda skulle kunna vara livräddande för vissa patienter. Sjukdomen är också svårdiagnosticerad och symtomen liknar många andra vanliga sjukdomar. Nya markörer som på ett tydligare sätt kan avgöra om patienten har drabbats av lungembolism skulle vara av stort värde.

Signalmolekylen kvävemonoxid (NO) verkar kärlvidgande i lungcirkulationen. Det har också föreslagits att NO är en viktig modulator för att luften i lungor på ett så effektivt sätt som möjligt ska möta blodflödet, d.v.s. optimera lungans gasutbytesfunktion. Halten NO som lungan producerar kan mätas i utandningsluften. Eftersom NO förefaller motverka de förändringar som uppstår vid lungembolism syftade det aktuella avhandlingsprojektet till att undersöka NO:s funktion vid lungembolism i experimentella djurmodeller.

Först undersöktes om halten NO i utandningsluften förändras vid lungembolism. Det visade sig att NO kraftigt steg i den akuta fasen vilket indikerar att mätning av NO i utandningsluften skulle kunna vara till hjälp när diagnosen lungembolism misstänks. Studier där halten NO mäts i utandningsluften hos människor som drabbats av lungembolism bör utföras för att undersöka om halten också hos dem stiger.

Vi fann också att den kroppsegna NO produktionen är starkt skyddande mot effekterna som inträffar vid lungembolism. Detta visar hur viktig signalmolekylen NO är för lungans funktion och indikerar att tillförsel av NO-donerande läkemedel skulle kunna vara av nytta vid denna sjukdom.

Vi utvecklade därefter nya NO-donerande läkemedel som mer specifikt verkar på lungans blodcirkulation med minimala effekter på systemcirkulationen (stora kretsloppet). Blodtrycksfall i systemcirkulationen vid lungembolism kan vara skadligt då det kan inverka negativt på blodflödet till hjärtat. Vi fann att tillförsel av sådan läkemedelskandidat vid lungembolism verkade starkt kärlvidgande på lungans blodcirkulation och därmed sänkte lungcirkulationens blodtryck och underlättade högerhjärtats arbete. Detta visar att kärlvidgande behandling vid lungembolism skulle kunna vara av stor nytta för patienten. Framtida studier syftar till att utveckla detta koncept samt att undersöka effekten av lungselektiva NO-donerande läkemedel vid andra tillstånd där lungcirkulationen är kraftigt påverkad.

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