NO Effect on Inflammatory Reaction in Extracorporeal Circulation

Ex vivo Studies

MIKA LAHTINEN
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Abstract

Nitric oxide (NO) is expressed in inflammatory tissues. However, NO effects are controversial in inflammation; NO is described as acting in a dose dependent manner and possess both pro-inflammatory and anti-inflammatory properties.

The present thesis explored the role of NO in relation to white blood cell (WBC) and protein system activation by foreign surfaces in simulated extracorporeal circulation (SECC) using human whole blood from volunteer donors. Three doses of NO, 40 ppm, 80 ppm and 500 ppm, were administered and an array of markers of WBC and protein activation were studied. Neutrophil degranulation was detected with myeloperoxidase (MPO), human neutrophil lipocalin (HNL) and lactoferrin (LF); eosinophil degranulation with eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO); and basophil degranulation with histamine. Furthermore, whole blood and WBC capacity to produce reactive oxygen species (ROS) were studied and cytokine release was measured with IL-1 and IL-10. Complement activation was measured with C3a and C5b-9 complex and contact system activation with FXIIa-C1INH, FXIa-AT, FXIa-C1INH and FXIa-AT.

NO increased neutrophil degranulation at all dose levels and 80 ppm NO increased basophil degranulation; whereas, NO exerted no effect on eosinophil degranulation, WBC subset counts, cytokine release or capacity to produce ROS. In addition, while increasing both specific and azurophil degranulation with 40 ppm, 80 ppm and 500 ppm, NO reversed the classical degranulation hierarchy with 500 ppm and azurophil degranulation became predominant. Furthermore, NO effect was greater with 500 ppm than with 80 ppm, indicating a dose response effect. The lack of iNOS mRNA expression in WBC and lack of L-NAME effect on degranulation and nitrite/nitrate production, together with absent increase in nitrite/nitrate in controls, excluded autocrine or paracrine regulation of degranulation. FXIIa-AT and FXIa-AT complexes increased and became predominant during early recirculation, whereas FXIIa-C1INH and FXIa-C1INH complexes were predominant at baseline but remained unaltered, suggesting contact system inhibition predominantly via AT. C3a and C5b-C9 increased. NO had no effect on either contact or complement system activation; however, 500 ppm NO shortened active clotting time.

In conclusion, the present data suggest that NO has a direct effect on neutrophil and basophil degranulation. Recognition of NO as an enhancer of degranulation may give access to new therapeutic tools for local and systemic inflammatory therapies; whereas, the identification of increased AT mediated inhibition of FXIIa and unchanged C1INH complexes presents new possibilities for therapeutic intervention in conditions such as hereditary angioedema and heart surgery.

Keywords: Cardiac surgery, extracorporeal circulation, inflammatory reaction, NO, degranulation, neutrophil, eosinophil, basophil, complement, contact system, coagulation

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Paper III. NO enhances basophil degranulation whereas antithrombin is primary inhibitor of FXIIa during inflammatory reaction. M. Lahtinen, J. Sanchez, G. Elgue, H. Khamis, J. Borowiec and P. Venge. (Submitted)

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<tr>
<td>SECC</td>
<td>simulated extracorporeal circulation</td>
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<tr>
<td>ECC</td>
<td>extracorporeal circulation</td>
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<tr>
<td>CPB</td>
<td>cardiopulmonary bypass</td>
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<tr>
<td>PMN</td>
<td>polymorphonuclear leukocyte</td>
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<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
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<td>HNL</td>
<td>human neutrophil lipocalin</td>
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<tr>
<td>LF</td>
<td>lactoferrin</td>
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<tr>
<td>ECP</td>
<td>eosinophil cationic protein</td>
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<tr>
<td>EPO</td>
<td>eosinophil peroxidase</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
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<tr>
<td>IL-1</td>
<td>interleukin 1</td>
</tr>
<tr>
<td>IL-10</td>
<td>interleukin 10</td>
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<tr>
<td>FXIIa</td>
<td>activated factor XII</td>
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<tr>
<td>FXIa</td>
<td>activated factor XI</td>
</tr>
<tr>
<td>C1INH</td>
<td>C1-esterase-inhibitor</td>
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<tr>
<td>AT</td>
<td>antithrombin III</td>
</tr>
<tr>
<td>C3a</td>
<td>activated complement component 3</td>
</tr>
<tr>
<td>C5b-9</td>
<td>total complement complexes (TCC)</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitric species</td>
</tr>
<tr>
<td>ACT</td>
<td>active clotting time</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NO$_2$</td>
<td>nitrogen dioxide</td>
</tr>
<tr>
<td>NO$_3^-$</td>
<td>nitrate</td>
</tr>
<tr>
<td>L-NAME</td>
<td>NG-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CL</td>
<td>chemiluminescence</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
</tr>
<tr>
<td>SOZ</td>
<td>serum opsonized zymosan</td>
</tr>
<tr>
<td>CPM</td>
<td>counts per minute</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger mRNA</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
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</table>
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1. Introduction

1.1 General background in heart surgery and inflammation

Extracorporeal circulation (ECC) is used in renal dialysis, heart-lung machine, liver transplantation and extracorporeal membrane oxygenation (ECMO). Oxygenation of blood and removal of carbon dioxide was first described in 1885, experimented on animals in the first half of 20th century and the pioneering clinical work with cardiopulmonary bypass (CPB) performed on humans in Philadelphia, Minneapolis and Stockholm in early 1950s. The clinical use of CPB was enabled by development of a method for extracting heparin in commercial quantities from beef lung and exploitation of protamine to neutralise heparin in the 1930s.

Modern cardiac surgery is performed either by allowing a CPB machine to serve as a pump-oxygenator i.e. pump blood to systemic circulation and oxygenate blood during cardiac arrest (on-pump) or with a beating heart technique without the assistance of a CPB machine (off-pump). On-pump surgery can be performed in total CPB i.e. all venous blood is returned through pump-oxygenator or partial CPB i.e. some part of the venous blood return is pumped by the heart to the lungs. On-pump technique with total CPB and cardiac arrest is mandatory during correction of intracardiac structures and is preferred over off-pump surgery in 75% of coronary artery bypass grafting operations (CABG) due to easier access to the cardiac structures during cardiac arrest: partial CPB is not used in routine cardiac surgery. Although on-pump and off-pump surgical techniques share anaesthesia and considerable chest wall and pericardial trauma, there are distinct differences. On-pump surgery is routinely performed with median sternotomy associated with large wound surface, whereas minimally invasive sternal or anterolateral incision is often used in off-pump surgery. Cardiac arrest results in global heart ischemia during on-pump surgery, whereas the beating heart experiences ischemia-reperfusion in the supply area of the revascularised vessel. Deprivation of pulmonary arterial circulation and cessation of ventilation results in relative lung ischemia and atelectasis of the lungs during on-pump surgery, whereas lungs are ventilated and perfused in off-pump technique. The foreign surfaces in CPB circuit triggering coagulation, use of
heparin-protamine, hemodilution and hypothermia are unique to on-pump surgery.

Heart surgery is associated with inflammatory reaction. Acute respiratory distress syndrome (ARDS) and systemic inflammatory reaction syndrome (SIRS) became recognised in the late 1960s and early 1970s and the resemblance of postperfusion syndrome after CPB to these conditions was observed. Several blood elements are activated during heart surgery: monocytes, neutrophils, eosinophils, basophils, platelets, complement system, contact system, coagulation, and fibrinolysis. The immediate postperfusion syndrome comprises tachycardia, tachypnea, hyperthermia, coagulopathy, oedema, vasoconstriction, and pulmonary and myocardial dysfunction. ARDS develops in 1.3% of CPB patients of which 53% die, and serum creatinine level increases in 7.7-11.4% of patients, a further 3.7% suffer acute renal failure, and 0.7-1.4% require postoperative dialysis with an overall mortality of 28-64%. Total CPB time, volume pumped through oxygenator and heart global ischemia time are risk factors for cardiac and pulmonary dysfunction, as well as for morbidity. Although most patients experience some degree of organ dysfunction immediately after CPB, CPB has also long-term effects in neuropsychological tests. Generally, complications occur more frequently when a patient is neonate, infant or elderly: infants accumulate fluid, whereas very elderly suffer lung complications.

The explanations for the cause of postperfusion syndrome have varied. Most frequently, neutrophil and platelet activation by foreign surfaces in CPB are claimed, whereas microembolism was observed in the 1960s, ongoing coagulation despite heparinisation in the 1970s, complement activation in the 1980s and endotoxaemia and cytokine release during the 1990s. Studies in SECC, animals and humans suggest that several assaults could contribute to the observed disruptions in cellular and protein subsystems. These include: neutrophil priming before CPB; administration of anaesthetic agents; sternal and pericardial wounds; foreign surface contact in CPB circuit and suction devices; endotoxaemia due to bacterial translocation in bowel; pooled pericardial blood and contamination of cardioplegia, cell saver and CPB devices; underlying atherosclerosis; and ischemia-reperfusion injury in myocardium and lungs. Partial CPB is tolerated for days and total CPB for hours and there is no convincing difference in inflammatory outcome or in neuropsychological complications between on-pump surgery with total CPB and off-pump surgery. This suggests that foreign surface contribution to the inflammatory reaction may be overestimated, whereas the roles of myocardial and lung ischemia-reperfusion injury as well as extent of sternal, pleural and pericardial trauma appear traditionally underestimated. Nevertheless, the additional insults associated with on-pump surgery with total CPB, compared to off-
pump surgery, could be crucial in high-risk groups such as elderly, neonates, infants and children.

The present task for research is to determine the relative importance of each insult on each patient group and quantify insults and activated mechanisms that need to be and can be attenuated. This thesis concentrated on the foreign surface-blood interactions in SECC and resembled conditions during on-pump cardiac surgery with CPB. Nevertheless, it is important to understand that several of the phenomena observed as a result of blood foreign-surface activation can also occur in a clinical situation as a response to other insults during cardiac surgery, such as anaesthesia, drugs, surgical wound, and ischemia of the heart and lungs.

1.1.1 Elements of cardiopulmonary bypass and simulated extracorporeal circulation

The basic components of the cardiopulmonary bypass are still the same as 50 years ago: oxygenator; tubings; venous reservoir; pump; and heat exchanger 36. The oxygenator is the largest area of foreign surface and allows the oxygenation of blood and removal of carbon dioxide in modern hollow fibre oxygenators across tiny pores in hollow fibres. In comparison, contact surface of the tubings made of polyvinyl or silicone rubber is minor. The venous reservoir drains blood from the patient’s right atrium by a gravity siphon storing the excess of blood volume during CPB. Thus, the blood draining to the venous reservoir from the heart, low in oxygen and high in carbon dioxide, can be delivered from the oxygenator, well oxidised and the excess carbon dioxide removed, through the aorta to the systemic circulation. An arterial roller pump determines the speed and volume of blood returned to the patient and generates a non-pulsatile flow. The heat exchanger regulates the temperature of the blood. Previously, a system with reduced tubing and without venous reservoir, thus reducing contact surface with blood, has been described 37.

SECC, used as an experimental model in this thesis, is created if the basic elements of CPB circuit are connected and blood is allowed to circulate without connecting a patient to the circuit. The model is well described in various studies on blood cells and protein systems 9,14,22,38. After foreign surface contact, plasma components adsorb instantly onto the foreign surface in concentrations different from those in the bulk plasma and form an immobile monolayer. Some proteins, for example fibrinogen, are selectively adsorbed and others, such as FXII and C3, undergo conformational changes to exposed reactive amino sites 2. Subsequently, platelets and granulocytes are activated. Every biomaterial has unique plasma binding geometry and properties that cannot be predicted by the physical and chemical characteristics of the biomaterial.
1.2 Granulocytes and cardiac surgery

Granulocyte response to perturbation of plasma membrane comprises an array of oxygen-dependent and oxygen-independent defence mechanisms. Oxygen dependent defence mechanisms involve the production of reactive oxygen species (ROS), and oxygen independent mechanisms include chemotaxis, phagocytosis, production of arachidonic acid metabolites and granule release.

1.2.1 Neutrophils

Polymorphonuclear neutrophil granulocytes (PMN) are the predominant infiltrating cell type during the cellular phase of acute inflammatory response. Generally, neutrophils are dormant, primed or activated. Priming is referred to as an amplification of granule release or production of ROS to secondary stimulus after first being exposed to a primary priming agent. Primers do not usually cause activation, however, sometimes neutrophils are primed by lower concentrations of a substance, which then at higher concentrations cause activation. Neutrophils respond to phagocytic and inflammatory stimuli by generation of ROS, production of cytokines and arachidonic acid derivatives, expression of adhesion molecules and release of granule contents into phagosome or to extracellular space.

Neutrophils contain two main types of granules, specific and azurophil. Azurophil granules contain an array of enzymes such as myeloperoxidase (MPO), lysozyme, elastase and cathepsin G. Specific granules comprise two-thirds of the granule population and contain several enzymes such as lactoferrin, NADPH-oxidase component cytochrome b558 and human neutrophil lipocalin (HNL), as well as extracellular matrix receptors referred to as adhesomes. In addition, there are gelatinase and secretory granules: gelatinase granules are small specific granules containing high amounts of gelatinase, have low lactoferrin content and contain adhesion molecules in the granule membrane; and secretory granules contain endocytosed material, such as alkaline phosphatase, and have adhesion molecules on the surface. Neutrophils secrete their granules independently and in hierarchical order to extracellular milieu. The hierarchy of mobilization is secretory vesicles, gelatinase granules, specific granules and azurophil granules i.e. the azurophil granule contents are the most difficult to mobilise. This is suggested as having functional significance: secretory vesicles contain substances needed in adhesion to the endothelium; gelatinase granules have further adhesion molecules and dissolve the first matrix barriers when migrating to the tissues; and then specific and azurophil granules participate in cytotoxic reactions and further dissolve extracellular matrix.
Despite having 10- to 20-fold lower RNA levels for cytokines than lymphocytes and monocytes, neutrophils are an essential source for cytokines, as they constitute the majority of cells in inflammatory focus 39. Neutrophils both respond to and produce pro-inflammatory cytokines: they are targets for IL-1α, IL-1β and IL-8; and produce TNF-α, IL-1α, IL-1β, IL-6 and IL-8, suggesting an auto-regulatory pathway 39,44. However, neutrophils also produce anti-inflammatory cytokines, such as IL-1RA and TGF-β; and the production of individual cytokines is determined by stimulatory conditions and regulated by other immunomodulatory cytokines, such as IFN-γ, IL-4, IL-10 and IL-13 from T-lymphocytes 39,44.

NADPH-oxidase complex provides neutrophils with the capacity to produce superoxide (O2-) and other ROS, whereas the existence of nitric oxide synthases (NOS) in human neutrophils and the subsequent capacity to produce NO and reactive nitrogen species (RNS) is controversial. Direct measurements of NO and its metabolites, such as nitrite (NO2-), and indirect methods such as platelet inhibition suggest the presence of NO production in neutrophils 45. Furthermore, eNOS mRNA 46, iNOS mRNA 47,48 and iNOS protein 46,47,49 are reported in neutrophils. However, negative studies reveal neither detectable NO 50, NO2- 51, iNOS mRNA 51 or iNOS protein 51. The interpretation of data in some studies is complicated by the capacity of neutrophils to generate NO independently of NOS from NO2- 52. When detected in neutrophils, iNOS protein is membrane bound 47, either located to perinuclear post-Golgi vesicles 47 or colocalized with MPO in azurophil granules 53. Bacteria, lipopolysaccharides (LPS), N-formyl-methionyl-leucyl-phenylalanine (fMLP) or multiple cytokines appear to serve as appropriate stimuli for iNOS induction 54.

Neutrophil counts decrease slightly during SECC 7,14,22,55 and initially during clinical heart surgery 27 indicating neutrophil adhesion to foreign surfaces or neutrophil entry to tissues. In clinical cardiac surgery, subsequent cell recruitment from bone marrow results in leukocytosis 27,56-58. However, myocardial ischemia causes leukocytosis and vascular surgery, thoracic surgery and beating heart surgery are associated with postoperative leukocytosis 29,34. This suggests that tissue injury and ischemia-reperfusion injury, instead of foreign surface contact, are major contributors to leukocytosis in clinical cardiac surgery.

1.2.2 Eosinophils

Eosinophils, traditionally considered as a defence against parasites, comprise less than 3% of cells in peripheral circulation. Eosinophilia typically results in fibrosis, fibrosis of endocardium in Löfflers syndrome, lung fibrosis in asthma and liver cirrhosis in scistosomiasis. Eosinophils contain primary and secondary granules both of which contain eosinophil peroxidase (EPO).
Primary granules (5%) are one-compartment granules containing Charcot-Leyden crystal protein and EPO. Charcot-Leyden crystals possess lyso-phospholipase activity suggesting a role in lipid and membrane metabolism. Secondary granules (95%) are bi-compartmental i.e. contain a central crystalline core and a matrix with an array of proteins. The major basic protein (MBP) and granulocyte monocyte-colony stimulating factor (GM-CSF) are located in the central crystalline core, whereas the rest of the proteins, such as EPO, eosinophilic cationic protein (ECP), MBP, eosinophil protein X (EPX) and cytokines, are located in the matrix compartment. Activated eosinophils produce cytokines such as IL-1α, IL-3 and IL-5. Moreover, eosinophils contain NADPH oxidase and iNOS and can thus generate ROS, NO and RNS. Eosinophil counts decrease during simulated and clinical dialysis and during clinical CPB.

1.2.3 Basophils
Basophils account for 0.5% to 1% of circulating leukocytes. Basophils phagocytose particles, process antigens and participate in responding against non-specific and specific stimuli. Basophils contain granules with histamine, cytokines and proteases complexed with proteoglycans (PG), such as heparin. The physiochemical properties of PG regulate the mediator release from mediator-PG complexes after exocytosis to extracellular milieu, thus influencing protease release rate and biological availability to tissues; for example, histamine is rapidly released from heparin. Basophils empty their granules more easily and more completely than neutrophils and eosinophils. Basophil iNOS expression and basophil counts during SECC or clinical CPB are not reported in literature.

1.3 Reactive oxygen species production
Oxygen undergoes partial reductions with subsequent reactive oxygen species (ROS) generation before being ultimately reduced to water.

$$
\begin{align*}
+e^- & \rightarrow O_2^- & +e^- & \rightarrow H_2O_2 & +e^- & \rightarrow OH^- & +e^- & \rightarrow H_2O \\
+2H^+ & \rightarrow -OH^- & +H^+ & \\
\end{align*}
$$

Reaction I

ROS comprises free oxygen radicals, superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radicals (OH), as well as a variety of their reaction products. Meanwhile, unpaired electrons provide free radicals with reactivity. $O_2^-$ has a mediate reactivity; $H_2O_2$ reacts slowly; OH and hypochlorous acids (HOCI) are strong oxidants; $O_2^-$ and $H_2O_2$ toxicities are low, and HOCI toxicity is somewhat controversial (see MPO discussion).
ROS are produced either endogenously or derive from exogenous sources, such as cigarette smoke. The endogenous ROS sources include: respiratory burst in phagocytes; mitochondrial respiratory chain due to leakage of electrons from electron transport chain; peroxisomes in nearly all eukaryotic cells; prostaglandin synthesis by lipoxygenases and cyclooxygenases; and degradation of purine derivatives (xanthine and hypoxanthine). The cellular ROS levels are regulated enzymatically by superoxide dismutases (SOD) and glutathione peroxidases (GPX), whereas extracellular and phagosome levels are regulated by SOD and peroxidases (MPO, EPO). In addition, antioxidants or scavengers in cellular membrane and cytosol can neutralise ROS.

The extracellular non-enzymatic anti-oxidant system comprises transferrin, caeruloplasmin, lactoferrin, albumin, haptoglobins, bilirubin, carotene, vitamin C, vitamin E, uric acid and acute phase proteins. The preventive anti-oxidants bind metals, such as copper and iron, whereas chain breaking anti-oxidants, such as albumin, act as scavengers and sacrifice their function through damage or consumption. The relative importance of enzymatic and non-enzymatic anti-oxidants depends on which ROS are generated, how and where they are generated, and what the target of the damage is. ROS and RNS act as second messangers and hormones for regulating gene expression and growth: low levels stimulate mitogenesis, somewhat higher levels cause transient or permanent growth arrest, and high levels cause apoptosis or necrosis. Excess of ROS or failure of mechanisms protecting against ROS cause damage to lipids, proteins and DNA resulting in altered membrane permeability, modified lipid-protein interactions, and active degradation products. The reactions are reversible or irreversible and the affected lipids, proteins and DNA are repaired, degraded or replaced.

NADPH-oxidase complex is located in both phagosome and plasma membrane, although several other cell types contain NADPH oxidase homologue (NOX), and is the electron source for the catalysis of O2 to O2\(^{2-}\) in phagocytes. The enzyme complex oxidizes NADPH on the cytoplasmic side of the membrane and transports electrons to O2 across the membrane, thus generating O2\(^{2-}\) either in the phagosome or in the extracellular milieu. In the secondary reaction, two molecules of O2\(^{2-}\) can spontaneously dismutate to H2O2 (Reaction I) or O2\(^{2-}\) can react with H2O2 to produce OH\(^-\) (II):

\[
H_2O_2 + O_2^{2-} \rightarrow OH^- + OH + O_2 (II)
\]

O2\(^{2-}\) is also enzymatically dismutated to H2O2 by inducible SOD in mitochondria (MnSOD), cytosol (CuZnSOD), peroxisomes (MnSOD, CuZn-SOD) or in extracellular milieu (ec-SOD):
Furthermore, H$_2$O$_2$ can also be formed via direct divalent reduction by glucose oxidase or by xanthine oxidase. H$_2$O$_2$ in turn generates extremely reactive OH$^-$ in the Haber Weiss reaction (II). To avoid excessive OH$^-$ generation, H$_2$O$_2$ is neutralised by:

- cytosolic or mitochondrial GPX (IV):
  \[ 2\text{GSH} + \text{ROOH} \rightarrow \text{GS-SG} + \text{ROH} + \text{H}_2\text{O} \] (IV)
- catalase in peroxisomes (V):
  \[ 2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \] (V)
- peroxidases (MPO, EPO) in phagosomes or in extracellular milieu (VI):
  \[ 2\text{H}_2\text{O}_2 \rightarrow 2\text{HOX} + 2\text{OH}^- \]
  \( +2\text{X}^- (\text{Cl}^-, \text{Br}^-, \Gamma, \text{SCN}^-) \)

O$_2^-$ reaction with MPO generates singlet oxygen (\(^1\text{O}_2^*\))$^{65}$, H$_2$O$_2$ reaction with MPO generates HOCl and reaction with EPO usually generates hypobromous acid (BrCl). HOCl in turn can undergo various reactions such as:

- with O$_2^-$:
  \[ \text{HOCl} + \text{O}_2^- \rightarrow \text{OH}^- + \text{O}_2 + \text{Cl}^- \] (VII)
- with H$_2$O$_2$:
  \[ \text{HOCl} + \text{H}_2\text{O}_2 \rightarrow \text{HOX}^* + \text{H}_2\text{O} + \text{Cl}^- \] (VIII)
- with amines$^{72,73}$:
  \[ \text{HOCl} + \text{R`RNH} \rightarrow \text{R`RNH} + \text{H}_2\text{O} \] (IX)
- with NO$_2^-$:
  \[ \text{OCl} + \text{NO}_2^- \rightarrow \text{NO}_2^- (\text{nitrate}) + \text{Cl}^- \] (X)
  \[ \text{HOCl} + \text{NO}_2^- \rightarrow \text{NO}_2^- + \text{Cl}^- + \text{OH}^- \] (XI)

Myocardial ischemia-reperfusion and foreign surface contact$^{74}$ cause acceleration of ROS production during cardiac surgery. Leukocyte capacity to produce ROS and human total plasma anti-oxidant capacity (TPAC), or total plasma antioxidative status (TPAS) comprising mainly of chain-breaking anti-oxidants, is used to measure ROS production and oxidant stress$^{75}$. However, changes in ROS production capacity and in anti-oxidative capacity in association with cardiac surgery are contradictory: enhanced$^{76,77}$ and reduced$^{57}$ isolated leukocyte ROS responses are reported during and after clinical CPB; and generation of ROS and products of ROS catalysed oxidation of lipids$^{74,78}$ and changes in blood levels of anti-oxidative capacity$^{79}$ are inconclusive. Lower urinary excretion of hypoxanthine, xanthine and malondialdehyde and lower levels of lipid hydroxyperoxides, protein carbonyls and nitrotyrosine are reported in off-pump surgery$^{34}$. However, administration of anti-oxidative therapeutics does not improve patient outcome in on-pump surgery$^{80}$ suggesting that ROS, RNS and RIS (reactive iron species) production may primarily regulate cell functions.
1.4 NO, nitric oxide synthases and reactive nitrogen species

Nitric oxide (NO) is a second messenger autacoid, paracrine substance, neurotransmitter and hormone exerting multiple physiological effects. NO is produced enzymatically by different nitric oxide synthases (NOS) generating l-citrulline, hydrogen and NO from l-arginine.

\[
l-\text{arginine} \rightarrow l-\text{citrulline} + H^+ + NO \\
NOS
\]

Constitutive (cNOS) calcium-dependent isoforms, type 1 NOS and type 3 NOS, are expressed in neuronal tissue and vascular endothelium, whereas inducible isoform (iNOS), also referred to as type 2 NOS, is a calcium-independent enzyme expressed in an array of cells, such as macrophages, eosinophils, liver cells, pulmonary cells and vasculature. iNOS is induced in most cells by hypoxia, inflammatory cytokines, such as TNF-\(\alpha\), IL-1\(\beta\), IFN-\(\gamma\), or bacterial products such as LPS. However, iNOS is also constitutively expressed in small intestine, lung epithelium, skin and nasal mucosa. An additional NOS subgroup, mitochondrial NOS, resembles type 1 NOS and regulates intracellular oxygen consumption. NOS requires NADPH and O\(_2\) as cosubstrates and flavin adenine mononucleotide (FMN), flavin adenine dinucleotide (FAD), tetrahydrobiopterin (BH\(_4\)), heme protein, calcium\(^{2+}\)/calmodulin, and possibly zinc\(^{2+}\), as cofactors. NOS produces O\(_2\)\(^-\) and H\(_2\)O\(_2\) instead of NO if l-arginine, cosubstrates or cofactors are absent or if NOS activity is inhibited. In addition, NO can be produced in vivo, without the involvement of NOS, directly from nitrite in granulocytes or cytochrome P450 catalysed oxidation of OH-l-Arg to NO and citrulline in the liver. NOS are inhibited by synthetic l-arginine analogues, such as NG-nitro-L-arginine methyl ester (L-NAME) and NG-nitro-L-arginine (L-NNA), each of which have their relative specificity to iNOS or cNOS.

iNOS produces nanomoles and eNOS picomoles of NO. NO biosynthesis is regulated at multiple levels including NOS transcription, posttranslational modification, protein expression and dimerization, enzyme modification and reaction kinetics. NO concentration and subsequently the function of NO scavengers also regulate iNOS and cross communication is suggested between cNOS and iNOS. Stimuli that inhibit cNOS often stimulate iNOS and thus cNOS is suggested as regulating iNOS by inhibition mediated by nuclear factor kappa B (NFkB). Some NO effects are mediated by the NO molecule itself, whereas, others are mediated by RNS generated in reactions between NO with O\(_2\) and ROS. The NO reaction pathway depends on when, where and how much NO is produced i.e. the production rate as well as conditions in extracellular milieu, such as pH, NO concentration, balance be-
tween NO, $O_2^-$, SOD, peroxidases (MPO, EPO) and catalase as well as co-factors of NOS that influence equilibrium 82.

1.4.1 NO reactions

1.4.1.1 NO reaction with various heme proteins
At neutral pH, NO readily reacts with various heme proteins but does not directly nitrate or oxidize lipid and protein targets 86.

- NO binding to oxyhaemoglobin yields methemoglobin and $NO_3^-$. $NO_3^-$ is a stable end-product of NO metabolism and a physiologic in vivo storage form of NO.
  \[ Hb(Fe-O_2) + NO \rightarrow \text{methHb(Fe(III))} + NO_3^- \] (XII)
- Guanylate cyclase is activated 400-fold by NO binding and generates cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP).
- NO acts both as a ligand and a substrate for peroxidases i.e. NO regulates MPO function 86 and NO is eliminated by MPO 87,88.
- Catalase oxidises NO and generates highly reactive nitrosonium (NO$^+$) 83

1.4.1.2 NO reactions with $O_2$ and $NO_2$

- NO autoxidation occurs primarily in lipid phase in physiological concentrations of NO and $O_2$, because NO and $O_2$ are 6-20 times more soluble in lipid layers than in aqueous solutions 83,86,87:
  \[ 2NO + O_2 \rightarrow 2NO_2 \text{ (nitrogen dioxide)} \] (XIII)
  $NO_2$ then dissolves 71:
  \[ 2NO_2 \rightarrow N_2O_4 \rightarrow NO_2^- + NO_3^- + 2H^+ \] (XIV)
- When enough NO is available, NO reacts with $NO_2$ generating dinitrogen trioxide ($N_2O_3$), followed by generation of $NO_2^-$ and nitrosonium (NO$^+$) with the ability to nitrosylate thiols 83.
  \[ NO_2 + NO \rightarrow N_2O_3 \rightarrow NO_2^- + NO^+ \] (XV)
  However, $N_2O_3$ may also undergo hydrolysis 89.
  \[ N_2O_3 + H_2O \rightarrow 2HNO_2 \] (XVI)
1.4.1.3 NO reactions with ROS, ONOO$^-$ and NO$_2^-$ reactions with ROS

- NO reacts readily with O$_2^-$ generating peroxynitrate (ONOO$^-$) $^{50}$. The relative availability of NO and O$_2^-$ determines the subsequent reaction pathway $^{90}$.

\[ \text{O}_2^- + \text{NO} \rightarrow \text{ONOO}^- \]  
(XVII)

ONOO$^-$ is a short-lived strong oxidant and is 2000-fold more effective than H$_2$O$_2$ in oxidizing thiols. Maximum ONOO production occurs when O$_2^-$ and NO are produced in equivalent amounts.

In normal pH, 80% is found as ONOO$, whereas ONOO$^-$ is protonated to peroxynitrous acid (ONOOH) under acidic conditions. ONOO$^-$ reacts with NO, O$_2^-$ $^{89}$ and CO$_2$ $^{71}$:

\[
\begin{align*}
\text{ONOO}^- + \text{NO} & \rightarrow \text{NO}_2 + \text{NO}_2^- \\
\text{ONOO}^- + \text{O}_2^- & \rightarrow \text{NO}_2 + \text{O}_2 + \text{NO}_2^- \\
\text{ONOO}^- + \text{CO}_2 & \rightarrow \text{Intermediates} \rightarrow \text{CO}_2 + \text{NO}_3^- 
\end{align*}
\]  
(XVIII, XIX, XX)

ONOOH decomposes:

\[
\begin{align*}
\text{ONOOH} & \rightarrow \text{OH}^- + \text{NO}_2 \quad (XXI) \\
\text{ONOOH} & \rightarrow \text{ONO}^-\rightarrow \text{NO}_3^- + \text{H}^+ \quad (XXII)
\end{align*}
\]

- NO reaction with H$_2$O$_2$ generates OH$^-$ $^{91}$ or $^1$O$_2$ $^{92}$.

- NO$_2^-$ can be oxidised by HOCl to either NO$_3^-$ or NO$_2$ $^{52,93}$ and can thus act as a HOCl scavenger:

\[
\begin{align*}
\text{OCl} + \text{NO}_2^- & \rightarrow \text{NO}_3^- + \text{Cl}^- \quad (XXIII) \quad \text{or} \\
\text{HOCl} + \text{NO}_2^- & \rightarrow \text{NO}_2^- + \text{Cl}^- + \text{OH}^- \quad (XXIV)
\end{align*}
\]

- NO$_2^-$ reaction with HOCl generates NO$_3^-$ which is able to scavenge O$_2^-$ $^{93}$:

\[ \text{NO}_2^- + \text{HOCl} \rightarrow \text{NO}_3^- + \text{Cl}^- + \text{H}^+ \]  
(XXV)

1.4.2 NO mechanisms of action

The chemical reactions define the mechanisms of action, which can be classified as direct or indirect. Direct mechanisms, occurring at low concentrations (< 1 µM), are mediated by transition metals. Indirect effects, occurring through reactive nitrogen species (RNS) $^{83}$, are mediated by high concentrations (> 1 µM). The reactions are also classified as classical-receptor mediated or redox-tone modulation, which comprises of a modulation of parts of the signalling pathway with the creation of a new baseline for sensibility to other stimuli. Direct NO interaction with heme iron in soluble guanylate cyclase and catalase are examples of classical receptor mediated effects. NO inhibition of cytochrome c oxidase and prostacyclin synthetase through non-
receptor mediated high concentration mechanisms involves oxidation (when one or two electrons are removed from the substrate); nitrosation (when NO+ is added to an amine, thiol, or hydroxyl aromatic group); or nitration (when NO2+ is added to a molecule). S-nitrosation and S-nitrosylation are comparable to phosphorylation. The relative specificity of S-nitrosylation is provided by presence of thiols in proteins, distance to ROS/RNS generation site and subcellular localisation of the target. High NO concentrations are often local and the effects are mediated by either direct or indirect mechanisms: in tissues distant from NO source, direct effects are predominant due to lower concentrations. Both NO and RNS can influence same enzymes and the true mediator, NO or RNS, and mechanism of action is not always clarified.

1.4.3 NO in clinics and in cardiac surgery

Various inflammatory conditions such as arthritis, asthma, systemic inflammatory reaction and sepsis are associated with iNOS upregulation. In blood cells, iNOS is expressed in eosinophils and monocytes, whereas neutrophil iNOS mRNA and protein expression has been both verified and denied. Myocardial iNOS is expressed preoperatively in children with congenital cyanotic heart diseases, in heart failure and in patients admitted for CABG and valve replacement. iNOS activity increases in lung epithelium during CPB. Furthermore, NO2−/NO3− generation is controversial in cardiac surgery: increase in NO2−/NO3− production is reported both in routine CABG and only in the subgroup of diabetics; NO2−/NO3− production is also denied.

NO is a known vasodilator and bronchodilator, although, its role in inflammatory reaction is more controversial. NO is suggested to have anti-inflammatory properties by modulating CD11/CD18 expression on the PMN surface and the expression of endothelial integrins, decreasing vascular permeability, inhibiting formation of O2•− in PMN by NADPH oxidase inhibition and acting as a scavenger of O2•− in the endothelial cell (NO reacts with O2•− three times faster than SOD). However, there is evidence that cytokines mediate myocardial contractile dysfunction in sepsis through the activation of either eNOS or iNOS and large amounts of NO are cytotoxic at high concentrations. In addition, NO is reported as either a protective or harmful agent during ischemia-reperfusion injury.

During therapeutic interventions, NO can be delivered to the patient: NO gas via a ventilator is used primarily to counteract pulmonary vasoconstriction and 1-80 particles per million (ppm) is usually administered to postoperative cardiac surgical patients. NO dose via a ventilator is limited by lung toxicity: that concentrations less than 50 ppm are not toxic, whereas 20 000 ppm is associated with bronchial and tracheal damage and methemoglo-
binemia. NO-containing chemical nitrovasodilators, such as nitroprusside and nitroglycerine, either spontaneously release NO in aqueous solution or react with tissue thiols, such as cysteine and glutathione, in smooth muscle cell layer and generate chemically unstable intermediates, S-nitrosothiols, that subsequently decompose with the liberation of NO. Cigarette smoke contains 400-1000 ppm, thus providing locally high pulmonary concentrations. NO is inactivated by heme-iron in erythrocytes and increasing methemoglobinemia is a sign of overdose: however, haemoglobin can also be considered as a NO carrier to ischemic sites.

1.5 Cytokines

Cytokines are a group of low molecular weight glycoproteins produced by an array of cells and exert biological effects on target cell surfaces via receptors. White blood cells act as both a source and a target for cytokines. Cytokines affect ROS production, granule release, adhesion molecule expression and other cytokine release. After an inflammatory stimulus, a cascade of events is initiated and mediators are both pleiotropic and redundant i.e. each mediator has more than one function, and mediators may overlap in their biological effects. Moreover, cytokine effects are not universally anti- or pro-inflammatory and there is synergy between the effects of different mediators. Each cytokine is capable of inducing the release of itself, other mediators and cytokines, and antagonists to its own activities too. Chemokines are cytokines that can specifically recruit discrete leukocyte populations.

Pro-inflammatory cytokines cause hypotension, fever, malaise, and weight loss. Pro-inflammatory cytokines are present preoperatively in some cardiac surgical patients, and increase after anaesthesia induction and heparin administration. Ischemia reperfusion of the heart and lungs, endotoxaemia and foreign surface contact trigger further cytokine production and release. Cytokine levels increase exponentially after 60 minutes of cross-clamping or longer ischemia times during transplantation, whereas exclusion of CPB and use of patients own lungs as an oxygenator appreciably reduces the pro-inflammatory cytokines. In fact, the myocardium and the lungs are considered as a major source of pro-inflammatory cytokines after organ reperfusion; however, the net effect on inflammatory cytokine equilibrium i.e. the balance of pro- and anti-inflammatory cytokines is to some extent controversial. Despite reported early postoperative pro-inflammatory states, patients exhibit a decrease in proinflammatory cytokines and immunosuppression intra-operatively, have predominantly anti-inflammatory cytokine release after declamping and a reduced capacity to synthesise cytokines during and after CPB. Furthermore, although higher postoperative expression of IL-1, IL-8, IL-10 and TNFα
mRNA in peripheral blood cells is observed on-pump, a comparison of studies of on-pump and off-pump does not provide a clear answer on cytokine balance. However, local cytokine tissue expression and levels may differ between organs, as well as between organs and plasma levels.

1.5.1 Interleukine-1 (IL-1)

IL-1α and IL-1β isoforms are released from monocytes, lymphocytes, neutrophils, NK cells and endothelium. Neutrophils express both IL-1α and IL-1β, but the quantity of IL-1β is up to 10 times more than for IL-1α. More than 80% of IL-1β is located intracellularly and relocates upon cellular activation to the outer surface of cellular membrane, from where it is released to the surrounding tissue by plasmin regulated processes. Although there is a difference in quantities, IL-1β is released in parallel to TNF-α and IL-8. IL-1 is regulated in different levels: IL-1α and IL-1β production and degradation, IL-1 converting enzyme (ICE), receptor agonist (IL-1RA) produced in different isoforms, and two surface-binding molecules, type I and type II IL-1 receptors (IL-1RI, IL-1RII). Each regulation level has its own control mechanisms.

IL-1β increases in simulated ECMO but not in SECC. Furthermore, IL-1β release is contradictory during clinical heart surgery: increase is reported both after anaesthesia induction and heparinisation but before CPB, as well as after CPB in cell lysates and in plasma; levels are reported unaltered during surgery or first 24 hours after CPB. The difficulty in detecting IL-1β is explained by fast elimination by the kidneys, short half-life or localised release from cellular surface in certain organs. However, large urinary and plasma IL-1ra increase suggests pathophysiological significance.

1.5.2 Interleukine-10 (IL-10)

IL-10 is produced by T-cell subsets, B-cells, monocyte/macrophages and eosinophils and its effects are mediated through specific receptors complexes. There are five IL-10 structural homologs: IL-19, IL-20, IL-22, IL-24 and IL-26. IL-10 inhibits cytokine production in T-cells, monocyte/macrophages and neutrophils. Although enhancing soluble TNF-α receptors and IL-1 receptor antagonist (IL-1RA) production, IL-10 is suggested to have a role in T-cell differentiation and to inhibit expression of iNOS in monocyte/macrophages.

Generally, IL-10 remains unchanged during anaesthesia induction, in thoracic surgery and in SECC. However, in clinical CPB, IL-10 increase occurs before IL-6 and IL-8, and studies show increase of IL-10 levels during early CPB, during CPB, after release of cross clamp, after
cessation of CPB, protamine administration and reperfusion, or postoperatively on the day of surgery. The peak value is reported both early during CPB, 10 minutes after cross clamp release, at the end of an operation or after an operation. The levels return to baseline within either 2-6 h after surgery or 24 hours after an operation. There are no statistically significant differences in IL-10 between off-pump or on-pump patients.

1.6 Degranulation products

N-acetyl-β-glucosaminidase, β-glucuronidase, elastase, lactoferrin, ECP and HNL increase during SECC and clinical CPB, whereas increase in histamine release is contradictory.

1.6.1 Peroxidases

Divided into 16 subfamilies, the myeloperoxidase family comprises several enzymes sharing a common 500 amino acid catalytic sequence capable of degrading H₂O₂. However, although myeloperoxidase (MPO) and eosinophil peroxidase (EPO) share an overall homology of 70% and principal mechanisms of reactions with redox transformations, they differ in their properties.

1.6.1.1 MPO and EPO reactions

MPO comprises 5% of neutrophil dry weight and in the resting state is in ferric form. The first product during respiratory burst, O₂⁻, has minimal antibacterial activity and dismutases to H₂O₂. The ferric form of MPO reacts rapidly with H₂O₂ or HOCl to compound I releasing water or chloride, respectively. Then, compound I oxidises: I⁻; Br⁻; Cl⁻; thiocyanate; H₂O₂; NO⁻; NO₂⁻; indole derivatives; and tyrosine. MPO primarily forms HOCl, whereas EPO forms HOBr and hypothiocyanate. HOCl reacts with Cl⁻, H₂O₂, O₂⁻ and NO₂⁻ to form Cl₂, ¹O₂, HO and NO₂Cl, respectively. These oxidants can chlorinate, oxidize, and nitrate proteins, lipids, nucleosides, and aminoacids. Chlorination occurs more easily than nitration and is the main reaction in phagocytosis, although both nitration and chlorination are catalysed by secreted MPO in extracellular milieu.

In reaction with H₂O₂, MPO can act both as a catalase generating water and as a peroxidase generating hypohalic acids, the former dominating intracellularly in the alkaline vacuole. The direct cytotoxic mechanism of action via HOCl is disputed by two opposing viewpoints. In the first, HOCl...
is suggested as directly activating granulocyte proteases or inactivating protease inhibitors, thus having an indirect cytotoxicity. The second argument takes an opposite position, and suggests that large amounts of degranulated proteins immediately consume all available HOCl, leaving the remaining HOCl concentration ineffective. Subsequently, HOCl would inactivate proteolytic enzymes.

1.6.1.2 MPO inactivation and regulatory functions

MPO is secreted to the microenvironment within 10 minutes of activation and once in the microenvironment, proteases, ROS and pH changes rapidly inactivate 40% of MPO. MPO becomes attached to biological membranes on various cells and thus provides neutrophils with the capacity to localise peroxidations, autoaugment degranulation, and increase macrophage ROS production and cytokine secretion. Furthermore, endothelium has binding sites for MPO and internalises MPO and the endothelial surface-bound MPO sustains enzymatic activity.

MPO catalytic action is modified by NO and MPO is considered as a consumer of NO, thus decreasing NO bioavailability to the tissues and attenuating vasorelaxation and bronchodilation. Furthermore, MPO is reported to generate NO from NO. HOCl in turn is rapidly scavenged by methionine, cystein, glutathione, tryptofan and taurine: NO, NO and NO serve as HOCl scavengers. Some of the chemical reactions for HOCl are reviewed under the subtitle ROS production.

1.6.1.3 MPO association with diseases

There is evidence that MPO is associated with atherosclerosis and coronary syndromes. MPO is a more sensitive marker for azurophil degranulation than elastase during ECC. Generally, MPO increases 4-18 fold during clinical cardiac surgery with values peaking two hours after cessation of CPB.

1.6.2 Human neutrophil lipocalin

Lipocalins as a group have a variety of functions, serving as carriers for pheromone, steroids, and NO, as well as serving as prostaglandin D synthase in brain. HNL is expressed in several tissues but is neutrophil specific in blood cells. HNL is located in neutrophils in specific granules: one fraction of HNL is free and another covalently linked with progelatinase B (MMP-9). HNL binds bacterial catecholate-type ferric siderophores and thus by binding iron executes antibacterial action. HNL serves as a marker for bacterial infections and as a prognostic factor in urine and plasma for renal injury during paediatric ECC. However, HNL function during inflammation is not understood.
1.6.3 Lactoferrin

Lactoferrin derives from several tissues but is neutrophil specific in blood \(^{147}\). Lactoferrin is located in specific granulae in neutrophils, and serves as a bacteriostatic agent by controlling free iron levels around neutrophils. However, lactoferrin can serve as cytotoxic agent by increasing membrane permeability, possessing serine protease activity and catalyzing OH production \(^{148}\). Furthermore, lactoferrin facilitates neutrophil adherence to vascular walls and downregulates pro-inflammatory cytokine production \(^{148}\). Lactoferrin increases two-fold in general thoracic surgery \(^{149}\), levels increase after 15 minutes of ECC and peak up to 15-fold during CPB \(^{10}\). In contrast to lactoferrin, which decreases in the postoperative period, MPO and elastase increase postoperatively \(^{10}\) and remain elevated 24 hours after operation, indicating augmentation of azurophil degranulation in the postoperative period unparalleled by specific degranulation. This cannot be due to faster lactoferrin elimination as the half-life of elastase-α-PI complex is 1 hour and the half-life of lactoferrin is 1.3-22 hours.

1.6.4 Eosinophilic cationic protein

ECP comprises some 30% of eosinophil proteins, but is also present in neutrophils and monocytes \(^{150,151}\). However, only eosinophils and monocytes are capable of synthesising ECP \(^{151}\), whereas neutrophils endocytose ECP from the surroundings and store ECP in azurophil granules \(^{150}\). ECP is cytotoxic to parasites, has antibacterial and antiviral activity, kills tumour cells and injures host tissue cells. Although the bactericidal and cytotoxic mechanisms are not fully clarified, perforin like activity, cytotoxic myocardial \(^{152}\) and pulmonary \(^{153}\) injury and cytostatic growth inhibition are suggested \(^{154}\), but ECP bactericidal Rnase activity is controversial. Eosinophilia is associated with thrombosis \(^{155}\) and there are several possible prothrombotic mechanisms: ECP enhances FXII activation \(^{155}\) and inhibits thrombomodulins’ anticoagulant activity \(^{156}\), ECP, MBP \(^{157}\) and EPO inhibit heparin action \(^{158}\), and eosinophils contain PAI-2 thus inhibiting urokinase. ECP is also reported to enhance fibrinolysis via plasminogen activation \(^{155}\) and inhibit FXII activation \(^{159}\). ECP increases during experimental and clinical dialysis and clinical cardiac surgery \(^{11,62,63}\).

1.6.5 Histamine

Histamine (2-(4-imidazolyl)ethylamine) is formed from histidine by histidine carboxylase predominantly in basophils and mast cells. Platelet, monocye, neutrophil and lymphocyte \(^{160}\) levels are lower (1/100 to 1/1000) than in basophils \(^{161}\). Histamine is complexed with proteoglycan-protein complex in granule matrix and dissociates from PG after exocytosis. Two to three percent of histamine is secreted directly into the urine, whereas the rest
is metabolised within minutes to methylhistamine or imidazole acetic acid: 4-10% of methylhistamine is secreted and the rest undergoes degradation. Histamine binds to H1, H2 and H3 receptors \(^\text{161}\); H1 receptors cause vasopermeability, vasodilation, and contraction of bronchial and gastrointestinal smooth muscle; H2 receptors mediate gastric acid secretion by parietal cells, mucus secretion at various sites and activation of endothelial cells to release PGI\(_2\) release; and H3 receptors mediate neurotransmitter release in the central and peripheral nervous systems. Depending on which receptors are activated, histamine may have both inotropic (H2-receptors) and cardio-suppressive effects (H1-receptors) \(^\text{162}\). Histamine regulates vasomotor tone, is arrhythmogenic \(^\text{63}\), and in patients with carcinoid tumors causes heart disease development. In addition, histamine inhibits secretion from cytotoxic lymphocytes, neutrophils, and basophils and enhances immunosuppression by lymphocytes.

However, histamine release in cardiac surgery is contradictory, histamine is reported to increase: during tissue trauma \(^\text{163}\); general surgery \(^\text{164}\); at anaesthesia induction in heart surgery \(^\text{13}\); thoracotomy \(^\text{12}\); at commencement of ECC \(^\text{13,164}\); during reperfusion \(^\text{164}\), or postoperatively \(^\text{165}\). However, histamine is also observed to decrease \(^\text{166}\) or be unaffected \(^\text{164,165,167}\) during the majority of CPB. Histamine release during CPB may be age dependent i.e. histamine releasing capacity increases with age \(^\text{166}\).

### 1.7 Contact system

#### 1.7.1 Structure of the system

Briefly, the plasma kinin-forming system consists of three plasma proteins: coagulation factor XII (FXII, Hageman factor), prekallikrein, and high molecular weight kininogen (HK) \(^\text{16,168,169}\). The interactions of these three proteins with coagulation factor XI on negatively charged surfaces are traditionally referred to as contact activation \(^\text{168}\). However, endothelial and neutrophil cell membranes \(^\text{170}\) provide the relevant surfaces in vivo and enzymatic digestion of FXII can generate biologically-active split-products in the absence of foreign surface. The tissues have comparable kinin-forming systems in which intracellular conversion of a prekallikrein to tissue kallikrein occurs by means of enzymes that are not well characterized \(^\text{168,169}\).

FXII can be activated by kallikrein, macromolecular complexes formed during an inflammation response, or proteins along cell surfaces or be autoactivated by negatively charged inorganic surfaces. Kallikrein activation of FXII generates quantitatively more FXIIa than autoactivation. Cleavage of FXII results in activated fragments with functional similarities and differ-
ences. α-FXIIa binds to surfaces, whereas β-FXIIa is a plasma phase protein. Both α-FXIIa and β-FXIIa cleave kallikrein and activate fibrinolysis. α-FXIIa cleaves FXI and facilitates cleavage of FXII to FXIIa in feedback loop, whereas β-FXIIa cleaves FVII and activates classical complement pathway: alternative complement pathway is activated by both α-FXIIa and β-FXIIa. FXIIa is regulated by plasma protease inhibitors C1-esterase-inhibitor (C1INH), antithrombin (AT) and α2-macroglobulin. In plasma and protein milieu, C1INH is considered as a main inhibitor of both α-FXIIa and β-FXIIa 168,169,171,172. FXIa in turn is predominantly inhibited by α1-antitrypsin or C1INH 173,174; whereas α2-macroglobulin and by heparin catalyzed AT contribute to a minor degree 173,174. The role of AT increases with increasing heparin dose 171 but C1INH remains as a main inhibitor of both FXIIa and FXIa 173,174 inhibitor at all heparin concentrations in plasma milieu.

1.7.2 Contact system and SECC

Foreign surfaces activate FXII, followed by generation of α-FXIIa and β-FXIIa 16,168,169. SECC results are contradictory: β-FXIIa-C1INH complexes remain stable, whereas free AT, free C1INH and kallikrein activity decrease slightly in one study 176; the majority of 3- to 12-fold increases 14,22,55 in kallikrein-C1INH complexes occur during first 2-20 minutes of SECC 14; and generation of kallikrein-C1INH complexes is denied during SECC in another study 177. Furthermore, heparin can interfere in complex formation by inhibiting C1INH-complex generation 178.

1.7.3 Contact system and clinical cardiac surgery

FXII 15,179 and kallikrein-kinin system 180-183 are activated before ECC commences, indicating activation either by tissue injury 184, heparin 180,185 or by leukocytes. Subsequently, bradykinin is generated in neonates, infants 186 and adults 15 in the pre-bypass period. Some results contradict during and after CPB: FXII activation is reported in both adults 15,187 and children 188 but also denied 179 in adults. A decrease in plasma total kallikrein, reflecting decrease in prekallikrein, is observed during CPB 183, whereas increase in kallikrein-C1INH-complexes is denied during or after clinical CPB 14. A decrease in postoperative prekallikrein is both detected 188 and denied 188,189. Although kininogen levels decrease 190, 191, it has been difficult to demonstrate consumption of kininogen either before or after commencing ECC 15,183. Bradykinin increases 4- to 20-fold in adults 15,183 and 1.5- to 8-fold in children 167, 186 during clinical ECC, having peak values during the first 10 minutes 183, 192 or at the end of the ECC 15. Kallidin, tissue derived bradykinin, increases only slightly during ECC, but are elevated 1.7- to 5.2-fold after ECC 183. The concentrations of the contact-system activation-products are not correlated to the length of ECC 193 and the net contribution of the
contact system to net hemostasis and distribution of activation of these systems remains somewhat perplexing 194.

1.7.4 Contact system, coagulation and fibrinolysis

Data indicate that the role of contact system activation in the initiation of internal coagulation pathway is overestimated in cardiac surgery. Deficiency in FXII, prekallikrein or HK does not result in a tendency to bleed 194, despite prolonged partial thromboplastin time. FXI deficiency results in haemorrhagic diathesis, F1+2 complexes are formed during CPB in spite of FXII deficiency 195,196 and thrombin-antithrombin (TAT) complexes are generated despite HK deficiency 197. Furthermore, TAT 15 and F1+2 complexes 179 are formed before commencing ECC and without parallel extensive activation of kininogen 15.

FXII is essential to fibrinolysis and thus to the equilibrium between coagulation and fibrinolysis 194. That is, no cross-linked fibrin d-dimers (XDP) were generated with FXII deficiency during CPB in one case report 195, thrombosis of all bypass grafts occurred in a case report with FXII deficiency after CPB despite high dose heparin 196, and XDP was produced in another case report with HK deficiency 197.

1.7.5 Contact system and inflammatory cells during ECC

Granulocytes and contact system interact: neutrophils 198,199 monocytes 198 and macrophages 200 express tissue prekallikrein mRNA 199,201 and protein 199 and secrete tissue kallikrein 198 and kinins; whereas, the exact cellular localisation of formed contact system proteins remain to be determined 198. Furthermore, activated granulocytes cleave prekallikrein and kininogen 202.

FXIIa and kallikrein 203 cause neutrophil degranulation and contact system inhibition reduces elastase release, despite ongoing complement activation 22,55. Although contact system activation is self-limited during early recirculation, neutrophil and eosinophil degranulation continues in a linear manner. Neutrophil surfaces can provide an environment for interactions for all components of the kinin-forming cascade, and enabling local kinin generation by binding FXII 170, FXI 170, HK and prekallikrein. The kinins formed can then act on neutrophils in an autocrine manner promoting degranulation 170, or act in a paracrine manner on endothelial cells by causing endothelial cell contraction to enhance neutrophil passage to the extracellular space. However, granule contents can act reciprocally on contact system: elastase inactivates FXIIa and prekallikrein 204; elastase and tryptase generate bradykinin from kininogen 202; ECP either inhibits 159 or enhances 155 FXII and kallikrein activation; heparin and chondroitin sulphate activate FXII 205; and serine proteases cleave and thus inactivate C1INH 206.
1.8 Complement system

1.8.1 Structure of the system

In brief, complement system together with its regulatory factors and receptors consists of more than 30 proteins and is a component of the innate immune system for eliminating pathogens, non-self antigens and altered host cells through opsonization, release of anaphylotoxins and cytotoxic actions. Its activation comprises three phases: recognition and activation of one or several of the complement pathways — classical complement pathway (CCP), alternative complement pathway (ACP) or the mannose-binding lectin pathway (MBL); C3 activation; and binding, amplification and assembly of the C5b-9 membrane attack complex. The cleavage products, anaphylotoxins C5a, C4a and C3a, bind to different receptors producing symptoms of inflammation by increasing vascular permeability, contracting smooth muscle, and triggering the release of vasoactive substances from granulocytes. In addition, C3a and C5a can cause cardiac dysfunction, and C5b-C9 complexes activate human cells at lower concentrations via sublethal injury.

1.8.2 Complement and SECC

Classical complement activation occurs slower than contact activation. Although classical pathway is activated through C1 by β-FXIIa and alternative pathway by both β-FXIIa and α-FXIIa, SECC studies demonstrate that inhibition of contact system activation does not block complement activation. Furthermore, the kinetics of contact system and complement activation differ. The majority of contact activation occurs during the first 2-20 minutes and does not occur in a time-dependent manner in either SECC or CPB, whereas in SECC C3a production occurs linearly after an initial steeper increase during the first 30 minutes. C5b-C9 increases 11-130-fold in SECC.

1.8.3 Complement and clinical cardiac surgery

Increased Ba/B ratio, C4d/C4 ratio and C1-C1INH complexes, together with elevation in C3a and C5a, and parallel consumption of C3 and C4, aggregation of IgM immunoglobulin, and C1q consumption, suggest activation of both alternative and classical pathways in on-pump surgery. Contribution of both pathways is further supported by uncontrolled activation of classical and common pathways in a patient with C1q deficiency.

Complement activation due to surgical trauma is somewhat contradictory: complement activation is reported and denied in general surgery, re-
ported 188,214 and denied 214,215 during thoracotomy for pulmonary surgery, and denied in aortic surgery 216. Furthermore, aneurysmectomy without CPB, but with extensive tissue damage, and patients with standard CPB are reported to produce equal levels of C3dg 217. However, complement activation is observed in cardiac surgery before commencing ECC: C4d/C4 ratio 209 and C3a 15 increase after median sternotomy and C4d/C4 and Ba/B ratio increase 209 after heparinisation, but before commencement of ECC. Although median sternotomy enhances complement activation compared to anterolateral thoracotomy, the three-fold increase after median sternotomy 29 is relatively minor compared to 7- to 250-fold increase in C3a and 11- to 130-fold increase in C5b-C9 in SECC 207,208. C3a production is higher in on-pump surgery than off-pump surgery in both adults 149, 215 and children 188 suggesting an essential role of CPB associated insults - myocardial and lung reperfusion, foreign surfaces, reversal of heparin with protamine and endotoxin release.

1.8.3.1 C3 and C3a

C3 is an acute phase reactant produced by the liver and decreases approximately 50%, in proportion to hemodilution, immediately after commencing CPB and is then stable during ECC 165, 189. The activated C3 product, C3a, increases 2- to 10-fold during cardiac surgery in adults 181, 217-219 and children 20, 188, 189, 220, 221, peaking at the end of operation 20, 181, 189, 218, 220, 221 or with a second peak in the postoperative period 218, 222, 181, 217, 221. C3a levels during and after ECC correlate to the preoperative C3a levels 218, length of bypass 20, 210 and organ dysfunction in adults 210, 223 and children 20. In children, C3a levels correspond only to 4% of total maximal C3a level, whereas, the C3 conversion reveals a 14% C3 conversion 220.

However, some results are contradictory. C3a correlation with complications has been difficult to reproduce both in adults 224 and especially in children 167, 220. C3a correlation to the length of ECC is reported 220, whereas C3d/C3 ratio correlation to CPB length is denied 189. C3d/C3 ratio increases during ECC 165, 189 and C3d/C3 ratio is higher with multiple organ failure 189, but there is no difference in C3d/C3 ratio early after institution of ECC 189. Although total C3 and C4 decrease more in children with capillary leak syndrome, there is no associated increase in C3d/C3 ratio or C5a 165: despite increase during ECC, C3a 167, C3d/C3 ratio and C5a 165 are lower with capillary leak. With ECC during liver transplantation, only minor complement activation occurs 215. Data thus suggest that factors other than blood foreign-surface contact could contribute to complement activation in vivo.

1.8.3.2 C5b-C9

C5b-C9 complexes are not statistically significantly elevated in surgery without ECC 217. However, C5b-C9 complexes increase 1.5 to 3.5 times during ECC and similar to C3a reach first peak at the end of ECC 217 or at
the end of operation \textsuperscript{181,218,221}, with a second peak up to 48 hours in the post-operative period \textsuperscript{181, 221}. In children, concurrent with decrease in C5b-C9, total haemolytic complement activity decreases during ECC, being suppressed until the third postoperative day \textsuperscript{220}.

1.8.4 Complement and inflammatory cells
Complement activates neutrophils, eosinophils and basophils; however, C3a induced neutrophil stimulation can also be secondary to eosinophil activation \textsuperscript{225}. C3a is a potent chemoattractant for human eosinophils and mast cells, whereas, C5a is chemotactic for neutrophils, monocytes and macrophages.

SECC studies reveal that inhibition of contact system reduces neutrophil degranulation despite simultaneous ongoing complement activation \textsuperscript{22,55}. Nevertheless, there are several documented complement-mediated effects on inflammatory cells during ECC: C3a and C5a mediate formation of platelet-neutrophil complexes \textsuperscript{207} via expression of neutrophil CD11b/CD18 \textsuperscript{207,226}, whereas, C5b-C9 complexes contribute to the formation of monocyte-platelet conjugates \textsuperscript{208,226} via expression of platelet CD62P. Moreover, recombinant antibodies against C5 reduce C5b-C9 formation and CD11b expression.

1.9 Coagulation
1.9.1 Coagulation system and heparin
In brief, there is equilibrium between both thrombosis and bleeding, which are regulated by blood flow, endothelium, platelets and coagulation factors. Platelets, monocytes and endothelium participate with various procoagulants and anticoagulants in total coagulation \textsuperscript{2}. There are two theoretical models for the function of coagulation protein system. In the cascade model \textsuperscript{227}, an external pathway is activated by tissue factor (TF) deriving from tissues, such as heart, blood vessels and blood cells, and results in cleavage of FVII, possible cleavage of FX, and acts as a cofactor for subsequent FVIIa-catalysed activation of FX and FIX. Internal coagulation pathway, in turn, is activated by FXIIa produced in the liver. Activation of external and internal pathway causes sequential cleavage of plasma zymogen–serine-proteases resulting in thrombin formation, which in turn generates fibrin. In an alternative modern model \textsuperscript{227}, external pathway is activated as described previously, whereas FXII and FXI are activated by the product of external coagulation pathway thrombin. In this model, FXII thus serves as a sustainer for TF-initiated coagulation cascade instead of initiating coagulation.
Heparin is used as an anti-coagulant as it inhibits internal coagulation pathway factors, FXI, FX, FIX and thrombin, by catalyzing AT. However, the role of AT and heparin in FXII inactivation is limited. FXIIa is primarily inactivated by C1INH \(^{171,175}\) and to a minor degree by AT in plasma milieu, whereas heparin does not enhance AT mediated inactivation of either α-FXII or β-FXII \(^{171,175}\) at heparin concentrations less than 32 U/ml. Subsequently, fibrinolytic system and plasma kinin-kallikrein system activation can occur despite full heparinisation \(^{181,228}\). In addition, heparin has several potentially parallel procoagulant effects: heparin stimulates FXII activation \(^{180,185}\) and inhibits C1INH \(^{178}\); although being able to cause thrombocytopenia and platelet dysfunction, heparin activates platelets through antibody-dependent and -independent mechanisms. Antibodies against PF4/heparin-complex can activate the external coagulation pathway by inducing monocyte TF expression after CPB \(^{229}\), thus possibly contributing to the procoagulant state often observed after heparin neutralisation in connection to CPB.

1.9.2 Coagulation and SECC

Foreign surfaces adsorb and activate both proteins and cells. FXII and kallikrein-kinin system are activated during the first five minutes of SECC \(^{14}\). TF, in turn, is induced in monocytes after 2-3 hours of SECC \(^{7}\). TF is also released from TF-containing-microvesicles in platelets \(^{230}\) after complex formation with neutrophils and monocytes \(^{226,231}\). ROS generation and release of serine proteases from granulocytes contribute to the activation of TF released from platelets \(^{230}\).

1.9.3 Coagulation and clinical cardiac surgery

Similar to disseminated intravascular coagulation (DIC), there are parallel opposing stimuli for both thrombosis and bleeding: monocytes, platelets and coagulation cascade are activated and thrombin formed promoting coagulation; whereas, platelet loss and dysfunction, heparinisation and ongoing fibrinolysis promote tendency to bleed \(^{179,232-234}\). The activated coagulation with thrombin generation occurs in approximately 20% of patients despite heparin doses that are 2- to 3-fold compared to treatment of other clotting disorders and activated clotting times over 400 s \(^{2}\). Increased TF \(^{235}\), FVIIa/VII ratio and F1+2 \(^{30}\) in pericardial blood and increased FXIIa \(^{179}\), F1+2 and TAT complexes \(^{15}\) in venous blood after sternotomy and pericardiotomy indicate that coagulation is activated in chest wounds before commencement of ECC. However, although F1+2 are detected in venous blood before ECC \(^{15,179}\), the increase is several-fold during ECC, peaking 20 minutes post-bypass \(^{179}\). Despite adequate heparinisation, the production of markers for fibrin \(^{179,232-234}\) and thrombin generation during ECC \(^{15,179,232-234}\) indicate ongoing procoagulant activity via external pathway. In addition, F1+2 production in patients with FXII deficiency \(^{195,196}\), absent parallel in-
crease in FXIIa and F1+2\textsuperscript{15, 179}, and F1+2 production before\textsuperscript{179, 236} or without parallel IXa generation\textsuperscript{195} support external coagulation pathway activity.

Fibrinolytic activity increases in pericardiotomy wound\textsuperscript{237} and in blood\textsuperscript{15, 17} both before CPB commences, during sternotomy\textsuperscript{15} and after heparinisation\textsuperscript{238}, and during CPB\textsuperscript{15, 17}; whereas, absent plasmin-α2-plasmin inhibitor complexes in SECC and clinical CPB contradict ongoing general fibrinolytic activity\textsuperscript{14}.

1.9.4 Coagulation and inflammatory cells

There are an array of possible interactions between granulocytes and coagulation system during CPB. Fibronectin degradation products cause neutrophil degranulation\textsuperscript{239}, whereas heparin effect on neutrophil degranulation is contradictory: heparin primes MPO release, especially in the clinical dose between 3 to 5 IU/ml\textsuperscript{240}, and inhibits elastase release\textsuperscript{241}. Furthermore, heparin may inhibit α1-proteinase inhibitor, a neutrophil elastase inhibitor, thus providing a milieu for enhanced proteolytic function. There is a reciprocal relationship between granule contents and coagulation: granule contents can affect coagulation by either acting as procoagulants or anti-coagulants. There are several examples of granule release acting as a procoagulant stimulus by either directly stimulating coagulation or inhibiting anti-coagulant mechanisms. ECP may directly enhance FXII-dependent coagulation activation\textsuperscript{155}. MPO\textsuperscript{242}, ECP, MBP\textsuperscript{157} and EPO can inhibit heparin action\textsuperscript{158}: a mixture of eosinophil granule proteins, specifically EPO\textsuperscript{158}, and MPO inhibit heparin/tryptase complex. ECP inhibits thrombomodulin’s anticoagulant activity\textsuperscript{156} and MPO reduces thrombin affinity to heparin-AT complexes by oxidising thrombin. Cathepsin G and elastase activate FX and FV on neutrophil surface\textsuperscript{245}. In addition, PF-4 from platelet α-granules can bind and thus inhibit heparin action. Alternatively, some granule proteins have anticoagulative or fibrinolytic properties: ECP can inhibit FXII activation\textsuperscript{159}; and elastase, cathepsin G\textsuperscript{244} and ECP\textsuperscript{155} can activate plasminogen to plasmin. All these interactions are potentially relevant during ECC when granule release from all granulocyte subsets is increased, but the net effect on coagulation is dependent on the prevailing circumstances in the tissues.
2. Aims

The general aim of this thesis was to study how exogenously administered NO affected white blood cell and protein system activation after blood exposure to foreign surfaces in ECC.

The specific aims were to study in SECC

I if NO addition affected leukocyte subset - neutrophil, eosinophil and basophil - degranulation

II if NO addition affected leukocyte ROS producing capacity and cytokine production

III if NO addition affected contact system and complement activation

IV if NOS-inhibitor (L-NAME) addition influenced neutrophil degranulation and nitrite/nitrate production

V if iNOS was expressed in leukocytes by foreign surface activation
3. Material and Methods

The studies conformed to the Declaration of Helsinki.

3.1 Human blood
Human whole blood (450 ml (Papers I, II, III, IV) was drawn from healthy (taking no medication) informed donors into a transfer pack containing no heparin (Paper I), 1 ml (Papers II, III) or 0.45 mL (Paper IV) of heparin (5000 U/ml). In Paper I, 0.4 ml of heparin was directly added to the circuits.

3.2 Extracorporeal circuit
Experiments were performed in SECC. The circuits were assembled in a standard manner with an infant hollow fibre oxygenator with integral soft cell venous reservoir (D701 Masterflo 34, Dideco, Mirandola, Italy). In each paper, two circuits were simultaneously primed with 250 ml of unheparinised blood coming from same donor and 50 ml of Ringer Acetate (Paper I) or approximately 500 ml whole heparinised blood from two different donors (Papers II, III, IV). The blood was recirculated at 0.5 l/min for 23 hours (I) or for 180 minutes (Papers II, III, IV, V). Blood temperature was maintained at 34°C (I) or 35°C (Papers II, III, IV). During the experiment, active clotting time (ACT) was monitored (Papers I, II, III, IV) and 2000 U heparin was added if ACT was < 400 seconds. To L-NAME circuits (Paper IV), 28 mg L-NAME (NG-nitro-L-arginine methyl ester) solution was administered.

3.3 Nitric oxide delivery
Control circuits were ventilated with a mixture of oxygen/air 40%/60% flowing at 2 l/min (Paper I), 0.4 l/min (Papers II, III) or 0.2 l/min (Paper IV). The NO group was ventilated with oxygen/air 40%/60% flowing at 2 l/min (Paper I), 0.3 l/min (Papers II, III) or 0.1 l/min (Paper IV). Flow of NO mixed with nitrogen gas (N₂) was adjusted to 0.15 l/min (Paper I) or 0.1 l/min (Papers II, III, IV) to reach a NO concentration of 40 ppm and NO₂ < 1 ppm (Paper I), 80 ppm (Papers II, III) or 500 ppm (Paper IV) and NO₂ < 4 ppm.
3.4 Blood sampling and biochemical measurements

In Paper I, there were eight different time points - before starting the circulation, 5, 10, 15, 30, 60, 120 and 23 hours, and in Papers II, III, IV, four different time points - pretreatment, 60, 120 and 180 minutes. The first sample (pretreatment) was drawn either directly from the circuit (Paper I) or from the transfer bag (Papers II, III). In Paper IV, pre-treatment was taken from both the transfer bag and, after priming but before starting ventilation or circulation, from the circuit. The subsequent samples were taken from the circuit. MPO (Papers I, II, IV), LF (Paper II) and HNL (Papers I, II, IV) served as markers for neutrophil degranulation, ECP and EPO for eosinophil degranulation (Paper III) and histamine for basophil degranulation (Paper III). Capacity of whole blood and isolated leukocytes to produce ROS was estimated by chemiluminescence (CL) (Papers I, II). Pro-inflammatory IL-1β and anti-inflammatory IL-10 were detected (Paper II). Complement activation was measured with C3a and C5b-9 complex (Paper III). Contact system activation was assessed with FXIIa-C1INH, FXIIa-AT, FXIa-AT and FXIa-C1INH (Paper III). ACT was monitored (Papers I, II, III, IV). In addition, blood cell counts were determined (Papers I, II, III, IV), methemoglobinemia recorded (Paper IV) and nitrite/nitrate measurements performed (Papers II, III, IV).

3.5 Measurements

MPO (Papers I, II, IV) and ECP (Paper III) were quantified with radioimmunoassay (RIA) and HNL with double RIA (Papers I, II, IV), whereas IL-1β, IL-10, LF (Paper II) and histamine (Paper III) were determined with ELISA and EPO with fluoroorimmunoassay (Paper III). ROS was estimated by CL after stimulating with either serum opsonized zymosan (SOZ) or phorbol myristate acetate (PMA) (Papers I, II). Two different chemiluminescent probes were applied, lucigenin detecting O2− and luminol detecting other ROS (Papers I, II). C3a was analysed according to previously described methods with mouse capture antibody (mAb 4SD17.3) to coat microtitre plates, followed by incubation with biotinylated rabbit anti-C3a and detection by addition of streptavidin conjugated HRP (Paper III). Soluble C5b-9 complexes were determined with anti-neoC9 mAB McaE11 coated wells, followed by polyclonal anti-C5 antibodies and detection with HRP-conjugated anti-rabbit immunoglobulin (Paper III). FXIIa and FXIa complexes were measured with a solid-phase ELISA according to previously described methods (Paper III). iNOS mRNA was detected in isolated leukocytes with Real Time PCR (Paper IV). NO2−, NO3− (Paper IV) and total NO2−/NO3− (Papers II, III) were detected with commercial kits. Cell counts were determined with an automatic cell counter (Papers I, II, III, IV).
3.6 Statistical methods

Statistical analysis determined if: the mean differed (1) between two groups ( Papers I, II, III, IV ), (2) over the time ( Papers I, II, III, IV ), and (3) if the average difference between the two groups increased from one time point to another (i.e. interaction effect) ( Papers II, III, IV ). A two-way repeated measures analysis of variance ( ANOVA ) was conducted ( Papers I, II, III ). In Paper IV, there were three groups and, subsequently, a one-way ANOVA (mean) and a Kruskal-Wallis test (median) were used separately to determine differences among the three groups. If there was a statistically significant difference, a Tukey’s Studentized Range test was determined how the mean differed between the groups. Because multiple tests were conducted, Bonferroni correction was applied ( Papers I, II, III, IV ). For the comparison of proportional MPO/HNL release ratio in Papers II and IV, a three-way repeated measure ANOVA was carried out. The factors in this analysis were time (0 min, 60 min, 120 min, and 180 min), group (Control, NO), and NO dose (500 ppm, 80 ppm). P<0.05 was considered as significant and 0.05<p<0.1 was referred to as a trend.
4. Results

4.1 Cellular response

Neutrophil granule release (Papers I, II, IV)

Paper I. (40 ppm of NO, 23 hours). MPO and HNL became elevated at 15 minutes and increased during 23 hours recirculation in NO (MPO 20-fold, HNL 15-fold) and control (MPO 23-fold, HNL 18-fold) circuits (Figures 1A & 1B). MPO and HNL release were enhanced by NO at 30 minutes and 120 minutes (pG<0.05) resulting in proportionally higher MPO and HNL secretion in NO circuits (Table 1).

Figure 1 A                      Figure 1 B

Figure 1. Data show concentration of MPO (1 A) and HNL (1 B) in extracellular fluid (Papers I, II, IV). NO enhanced MPO release at 40, 80 ppm and 500 ppm. Although NO significantly enhanced HNL release at 40 ppm, there were only trends for enhanced HNL secretion at 80 ppm and 500 ppm.

Table 1. Proportional HNL, MPO, ECP, EPO and histamine secretion (Papers I, II, III, IV). In line with absolute values and MPO/HNL release ratio, the proportional degranulation of MPO, HNL, ECP and histamine were higher in NO circuits than in controls (Figures 1 and 2). However, there was no difference in proportional EPO release between NO circuits and controls.
Paper II. (80 ppm NO, 180 minutes). MPO, HNL and LF increased in NO (MPO 7.5-fold, HNL 5-fold, LF 28-fold) and control (MPO 3-fold, HNL 3-fold, LF 14-fold) circuits. NO enhanced MPO release ($p_{G120\text{ min}}=0.017$ and $p_{G180\text{ min}}=0.008$) and there was a trend for NO enhanced HNL ($p_{G120\text{ min}}=0.075$ and $p_{G180\text{ min}}=0.052$) and LF ($p_{G180\text{ min}}=0.07$) release. There was a significant interaction effect for MPO ($p_{I}<0.0001$), HNL ($p_{I}=0.004$) and LF ($p_{I}=0.03$) (Figures 1A & 1B). Proportional MPO and HNL secretion were higher in NO circuits (Table 1).

Paper IV. (500 ppm NO, L-NAME, 180 minutes). MPO and HNL increased in NO (MPO 20-fold, HNL 11-fold, control (MPO 3-fold, HNL 3-fold, L-NAME 8.5-fold, HNL 5.5-fold) circuits). MPO release was enhanced by NO compared to L-NAME circuits at 60 minutes ($p_{C}=0.046$), and compared to L-NAME and control circuits at both 120 minutes ($p_{C}=0.02$) and 180 minutes ($p_{C}=0.01$). For HNL, there was a trend for higher secretion in NO circuits ($p_{G60\text{ min}}=0.07$, $p_{G120\text{ min}}=0.11$, $p_{G180\text{ min}}=0.12$) and a trend for interaction effect ($p=0.053$) (Figures 1A & 1B). Furthermore, proportional MPO and HNL secretion were higher in NO circuits (Table 1).

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To plot the predominant type of neutrophil degranulation, azurophil or specific, at every time point separately in the NO circuits and controls, the proportional secretion of MPO and HNL was calculated (Table 1) and the ratio between proportional secretion of MPO and HNL was formed (Figure 2A, 2B, 2C). To better visualise NO effect on proportional MPO/HNL release ratio, the behaviour of difference in the proportional MPO/HNL ratio be-
between NO circuits and controls was plotted (MPO/HNL ratio_{NO} - MPO/HNL ratio_{Control}) (Figure 2 D).

Figure 2A, 2B and 2C. Data show ratio of proportional MPO/HNL release in NO and control circuits. The proportional MPO/HNL release ratio increased significantly with 80 ppm (p=0.02) (Figure 2B) and 500 ppm (p=0.02) (Figure 2C). Figure 2D. Data show the difference in proportional MPO/HNL release ratio (MPO/HNL ratio_{NO} - MPO/HNL ratio_{Control}) between NO and controls. The increase in difference in proportional MPO/HNL release ratio between NO circuits and controls was significant with 80 ppm (p_{G}<0.0001) and 500 ppm (p_{I}=0.024), whereas with 40 ppm NO there was a trend for increase in difference between NO circuits and controls (p_{I} = 0.092).

The reversal of secretion from HNL dominated (specific degranulation) to MPO dominant (azurophil degranulation) during recirculation was demonstrated by the behaviour of the proportional MPO/HNL release ratio: the proportional MPO/HNL release ratio for the controls remained relatively level over time and the values were < 1 at 180 minutes and earlier; whereas, the proportional MPO/HNL release ratio for the NO circuits steadily increased over time (80 ppm, 500 ppm) and was > 1 with 500 ppm. With 80 ppm NO, the MPO/HNL ratio was higher in NO circuits than controls at 180 minutes (p_{G}=0.02). With 500 ppm NO, the MPO/HNL ratio was higher for NO circuits than for controls and L-NAME circuits at 60 (p_{G}=0.03) and 180 minutes (p_{G}=0.02). There was no significant difference between controls and
L-NAME circuits at any time point. The proportional MPO/HNL release ratio increased steeper over time in 500 ppm NO circuits than in controls and L-NAME circuits ($p_I=0.01$). Furthermore, there was a significant increase for difference in the proportional MPO/HNL ratio over time between NO circuits and controls at 80 ppm NO ($p_I<0.0001$) and 500 ppm ($p_I=0.024$); whereas, there was a trend for increased difference in the proportional MPO/HNL ratio between NO circuits and controls at 40 ppm ($p_I=0.092$). The difference in proportional MPO/HNL release ratio between NO circuits and controls increased significantly more over time at 500 ppm NO than at 80 ppm NO ($p_I=0.037$) (Figure 2D).

**Eosinophil granule release (Paper III)**

ECP increased in NO (7-fold) and control circuits (4-fold) ($p_G<0.05$, $180$ min) and $p_I=0.01$), translating to higher proportional ECP release in NO circuits (Table 1). EPO increased in NO (4.7-fold) and control circuits (4.6-fold), but EPO release was not affected by NO ($p_G=0.72$ and $p_I=0.76$) (Table 1).

**Basophil release (Paper III)**

Histamine increased in NO (700-fold) and control circuits (250-fold) ($p_G<0.05$, $180$ min and $p_I=0.005$), translating to higher proportional histamine release in NO circuits (Table 1).

**ROS production capacity (Papers I, II)**

Paper I (40 ppm, 23 hours) (Peak values (Paper I) or area under curve (AUC) (Paper II) were given). Leukocyte $O_2^-$ production capacity was unchanged with PMA at 120 minutes but decreased with SOZ stimulation. Whole blood $O_2^-$ producing capacity remained relatively stable during the first 120 minutes, but decreased at 23 hours. Leukocyte other ROS production capacity decreased, whereas whole blood other ROS producing capacity increased during the first 120 minutes, followed by a decrease at 23 hours. There was no consistent difference between the NO circuits and controls.

Paper II (80 ppm NO, 180 minutes). Leukocyte $O_2^-$ producing capacity increased during the first 120 minutes with PMA stimulation and then decreased under baseline ($p_T=0.01$), whereas with SOZ stimulation it decreased after initial elevation at 60 minutes ($p_T=0.0003$). Whole blood $O_2^-$ production capacity decreased steadily over time with SOZ stimulation ($p_T<0.0002$). Leukocyte capacity to produce other ROS decreased at 180 minutes with both PMA ($p_T<0.0002$) and SOZ ($p_T=0.006$), whereas whole blood capacity to produce other ROS increased at 60 minutes and remained elevated compared to baseline at 180 minutes ($p_T<0.0002$). There was no consistent difference between the NO circuits and controls.
Cytokine release (Paper II)
Paper II (80 ppm NO, 180 minutes). IL-1β levels remained unchanged over time, whereas IL-10 levels increased ($p_{T}=0.007$) after an initial decline. NO did not affect IL-1β or IL-10 levels.

Blood cell counts (Papers I, II, II, IV)
Paper I (40 ppm, 2000 U heparin, 23 hours). White blood cell (WBC) counts decreased at 30 minutes and later ($p_{T}=0.05$). NO circuits had lower WBC count ($p_{G}<0.05$) at the end of the experiment.
Paper II (80 ppm, 5000 U heparin, 180 minutes). WBC, neutrophil and monocyte count decreased ($p_{T}<0.0002$). Platelet count decreased during the first 60 minutes where after it remained stable. Haemoglobin and lymphocyte count increased over time. There was no statistically significant difference ($p_{G}>0.05$) between the groups.
Paper III (80 ppm, 5000 U heparin, 180 minutes). There was no significant change in eosinophil ($p_{T}=0.98$) or basophil ($p_{T}=0.654$) counts and there was no statistically significant difference between NO circuits and controls ($p_{Geos}=0.725$ and $p_{Gbas}=0.22$).
Paper IV (500 ppm, 2250 U heparin, 180 minutes). WBC count decreased over time but there was no significant difference among the groups ($p_{G}=0.16$). Neutrophil count decreased over time ($p_{T}<0.0001$) and appeared higher in controls at the end of experiment ($p_{T}=0.052$). Lymphocyte count was higher for controls than for L-NAME and NO circuits ($p_{G}=0.04$): the difference was significant even at baseline. Eosinophil count decreased during the first 60 minutes ($p_{T}=0.0003$) where after it remained stable. Basophil count was unchanged over time and there was no difference among the groups. The mean platelet count decreased from pretreatment to 60 minutes ($p_{T}=0.008$) but there was no statistically significant difference among the three groups ($p_{G}=0.27$).

4.2 Protein system response

Complement activation (Paper III)
Paper III (80 ppm NO, 5000 U heparin, 180 minutes). C3a increased steadily ($p_{T}<0.0001$) in NO circuits (20-fold) and controls (18-fold)($p_{G}=0.2$ and $p_{T}=0.6$). Similarly, C5b-C9 complexes increased ($p_{T}<0.0001$) steadily in NO circuits (13-fold) and controls (15-fold)($p_{G}=0.3$ and $p_{T}=0.5$). NO did not affect C3a or C5b-C9 levels.
Contact activation (Paper III)

**FXIIa complexes**

At baseline, FXIIa-C1INH complexes were predominant (66%) compared to FXIIa-AT complexes (34%), whereas FXIIa-AT complexes (83%) became dominant during recirculation (compared to 17% FXIIa-C1INH). The decrease in FXIIa-C1INH complexes was not significant (p>0.6). In contrast, FXIIa-AT complexes increased 8-fold (p=0.001). There was neither significant difference between control and NO circuits nor interaction effect (p>0.6) (Figure 3).

![Figure 3](image)

*Figure 3.* Data show that majority of contact system activation occurred during the first 60 minutes and FXIIa inactivation went via AT complexing. NO had no effect on contact activation.

**FXIa complexes**

Only minor FXIa complexing occurred: at baseline, no FXIa-C1INH complexes were present, whereas 38% of circuits had FXIa-AT complexes. At the end of the experiment, 87% of circuits revealed non-detectable FXIa-C1INH complexes, whereas 88% of circuits had detectable FXIa-AT complexes. The increase in FXIa-C1INH complexes was not significant, whereas FXIa-AT complexes increased (p=0.005) 6-fold in NO and 10-fold in control circuits (p>0.6).

**Coagulation studies (Papers I, II, III, IV)**

Paper I (40 ppm, 2000 U heparin, 23 hours). ACT was measured at each time point but not registered in records.

Papers II and III (80 ppm, 5000 U heparin, 180 minutes). ACT was recorded unchanged at 60 minutes.

Paper IV (500 ppm, 2250 U heparin, 180 minutes). ACT decreased at 120 minutes in NO circuits (622±330 seconds, p=0.06), L-NAME circuits
(920±158 seconds, \( p_T = 0.34 \)) and in controls (826±188 seconds, \( p_T = 0.06 \)), (\( p_G = 0.26 \)).

4.3 iNOS and NO metabolites

iNOS mRNA (Paper IV)
There was neither iNOS expression nor difference between the groups for iNOS expression i.e. iNOS expression was similar to background in leukocytes at all time points and in all groups.

Nitrite and nitrate (Papers II, III, IV)
Papers III and III (80 ppm, 180 minutes). Total NO\(_2^-\)/NO\(_3^-\) remained unaltered in controls. Total NO\(_2^-\)/NO\(_3^-\) increased and was higher in NO circuits at each time point after initiation of circulation (\( p_G < 0.0002 \) and \( p_I < 0.0001 \)). Paper IV (500 ppm, 180 minutes). NO\(_2^-\) and NO\(_3^-\), measured separately, increased in NO circuits compared to controls and L-NAME circuits (\( p_{G(NO_2^-)} = 0.0008 \), \( p_{G(NO_3^-)} = 0.0006 \)). NO\(_3^-\) increased 20-fold in a linear manner, whereas NO\(_2^-\) increased 60-fold during first 60 minutes, peaked at 120 minutes and remained relatively stable during the rest of recirculation. There was no difference between L-NAME circuits and controls.

Methemoglobinemia
Paper IV (500 ppm, 180 minutes). In the NO circuits, 5.6 % was highest detected level at 180 minutes.
5. Discussion

General and study limitations

Cigarette smoke contains 400-1000 ppm NO and 1-80 ppm NO \(^{104}\) is used successfully to counteract pulmonary vasoconstriction in humans, whereas 40-100 ppm NO does not counteract platelet activation in human trials \(^{246,247}\). In the present series of experiments, 40 ppm was lowest and 500 ppm was the highest dose used, thus being representative of doses in vivo. Methemoglobinemia was at highest 5.6 % in 500 ppm NO circuits and in line with animal experiments \(^{288}\), being well below the 70% considered as lethal and less than the 15% considered as the symptomatic limit. However, NO \(^{249}\) in combination with SECC, could theoretically decrease cell viability and thus influence the applicability of the results from the present thesis to clinical conditions with increased NO production or NO administration.

SECC is used as a model for studying the foreign surface activation of blood elements and effects of pharmaceutical substances thereon \(^{9, 14, 22, 38}\). The use of an infant hollow fibre oxygenator, circuit temperature of 34-35°C, and flow rate of 0.5 l/min are representative of flow conditions in a 2.75 kg infant when no active cooling is used. The last sampling time at 23 hours in Paper I was unphysiologic, whereas sampling times until 120 minutes in Paper I and 180 minutes in Papers II, III, IV were comparable to clinical CPB. However, the SECC model has some limitations, the results cannot be directly transferred to a clinical situation, as blood from healthy donors instead of congenital heart disease or angina patients was used. New cells could neither enter the circuit from bone marrow and nor could cells escape to the tissues. There was no interaction between blood cells, endothelium and tissues, and surgical trauma to the sternum and pericardium, ischemia-reperfusion injury, and tissue metabolism of degranulation products were absent.
5.1 Cellular response

5.1.1 Granulocyte degranulation

**Neutrophil degranulation**

The difference in absolute levels and proportional secretion, both at baseline and during recirculation, in the whole blood experiments in Papers II and IV could be due to a different heparin dosage, the handling of bags, the different temperatures in the circuits and different ventilation of the circuits. The lower absolute values in Paper I could be explained by the division of the contents of one transfer bag into two circuits and haemodilution with crystalloids. Furthermore, the higher ventilation rate and lower temperature together with substantially lower volume in the circuit due to more frequent blood sampling during early recirculation exerted a greater mechanical stress on leukocytes. Subsequently, the results in Paper I were not comparable to the results in Papers II, III and IV.

That MPO release increased after 15 minutes in Paper I was in line with a previous test tube experiment. The observed 5.5-fold increase in MPO in controls in Paper IV was slightly higher than the 3-fold increase in Paper II, but in line with 4- to 18-fold increase in MPO in clinical heart surgery. NO enhanced MPO release by 7.5-fold with 80 ppm (Paper II) and 20-fold with 500 ppm (Paper IV). Similar to MPO, the 5.5-fold increase in HNL in controls in Paper IV was was slightly higher than the 3-fold elevation in HNL in controls in Paper II, whereas, HNL elevation was lower in Papers II and IV than 20-fold increase in HNL after clinical paediatric heart surgery. NO enhanced HNL release by 5–fold with 80 ppm (Paper II) and 11–fold with 500 ppm NO (Paper IV).

Generally, neutrophil specific granule and azurophil granule exocytosis can occur independently: minor stimuli cause specific granule release, whereas azurophil granules are hardly mobilised. In the present series, HNL and MPO degranulation occurred in a linear time-dependent manner. In Papers II and IV, there was stronger statistical evidence for NO enhancement of MPO than for HNL release, suggesting that NO preferably caused azurophil degranulation. Preferential azurophil degranulation by NO was further suggested by the proportional MPO secretion exceeding the proportional HNL secretion with 500 ppm NO (MPO 61%, HNL 37%) in Paper IV; whereas, the proportional HNL release exceeded the proportional MPO release in controls (HNL 25%, MPO 12%) in Paper II, (HNL 22%, MPO 18%) in Paper IV and in NO circuits with 80 ppm (HNL 37%, MPO 29%) in Paper II. The subsequent formation of the ratio of proportional MPO/HNL release in Papers II and IV statistically verified the change in the MPO/HNL release relationship during recirculation in NO circuits. Moreover, the increase in difference in proportional
MPO /HNL release ratio between NO circuits and controls was significantly faster with three-way repeated measures ANOVA analysis for 500 ppm than for 80 ppm at 180 minutes, verifying statistically the dose response effect on proportional MPO/HNL release ratio. Increasing proportional MPO/HNL release ratio with exogenous NO administration and a reversed degranulation hierarchy with high dose exogenous NO i.e. the higher HNL release at baseline reversed, in Paper IV, to MPO-predominant secretion during recirculation, are novel observations.

The present results concurred with early observations that an increase in cGMP and administration of l-arginine increase degranulation in stimulated neutrophils, but contradict some of the later studies using NO donors. However, the validity of the negative studies can be questioned, as the active compound of the NO donor used also inhibits intracellular free calcium. Compared with a previous study with heparin-coated SECC, the discrepancy in NO effect on neutrophil degranulation with increasing NO doses in the present series suggests a possible difference in leukocyte activation during contact with heparin-coated circuits, although the exact cause of the discrepancy remains to be determined.

The effects of NO on neutrophil degranulation are important findings for understanding neutrophil physiology and indicate that NO, in addition to serving as a ligand and substrate for MPO, also regulates MPO secretion in a dose dependent manner. This may in turn have consequences for the understanding of pathophysiology in several conditions. First, MPO levels are not just a marker for degranulation but are also associated with the risk of complications in paediatric cardiac surgery and in acute coronary syndromes, whereas HNL is a good predictor of renal failure in paediatric cardiac surgery. Second, cigarette smoke contains up to 400-1000 ppm NO and in the present study, 500 ppm NO primarily caused azurophil degranulation with MPO release. Thus, smokers have elevated MPO concentration in plasma and neutrophils, and as NO and MPO derived reactive intermediates are present in atherosclerotic plaques, it is probable that high NO dose in cigarette smoke is an important causative agent for atherosclerosis and possibly also for emphysema. Third, these results could have clinical relevance for the treatment of several diseases with NO gas or nitro donors, and for the development of future therapeutics for asthma, sepsis, transplant rejection, heart failure and ischemia-reperfusion, in all of which iNOS is induced and NO produced in parallel with ongoing granulocyte exocytosis. Fourth, NO could be advantageous in host defence against bacterial infections and tumours but unfavourable during sterile inflammations.

Although NO mediated intracellular mechanisms are the most probable cause of enhanced degranulation, it is important to recognise that increased MPO can autoaugment its own degranulation via CD11/CD18 binding.
MPO reacts rapidly with $H_2O_2$ or HOCl produced during respiratory burst and forms compound I $^{127}$, which in turn together with NO, $O_2^-$ and HOCl form new reactive intermediates chlorinating, oxidizing, and nitrating proteins, lipids, aminoacids $^{257}$ and nucleosides $^{137}$ with theoretical capacity to cause degranulation. In addition, MPO generates NO$_2$ from NO$_3^-$ $^{258}$, which in turn is known to cause granulocyte degranulation $^{259}$.

**Eosinophil degranulation**

The study confirmed previous results that ECP is released after foreign surface contact $^{11,62,63}$, whereas, the observation that NO enhanced ECP release is not previously described. However, the proportional ECP secretion in controls (3%) and NO circuits (5%) was low compared to degranulation associated with adhesion to serum coated Sephadex bead (15%) $^{260}$. Theoretically, parallel regulation of ECP and MPO release from eosinophils and neutrophils, or selective ECP secretion from neutrophils or eosinophils, could have occurred. The co-release of ECP and MPO from neutrophils was suggested by a combination of parallel ECP and MPO release in Papers II (3.5-fold in controls and 7.5-fold in NO circuits) and III (3-fold in controls and 7-fold in NO circuits) and the presence of ECP in neutrophil azurophilic granules $^{150}$. This was further supported by a similar magnitude of relative secretion for ECP (controls 12%, NO circuits 25%) and MPO (controls 12% NO circuits 29%), if it is considered that all ECP derived from azurophil granules in neutrophils and monocytes instead of from a pool of granulocytes, including eosinophils.

Subsequently, EPO analysis was performed to assess the true magnitude of eosinophil exocytosis and NO effect thereon. The results revealed a low degree of eosinophil degranulation but absent NO effect on eosinophil degranulation, verifying that ECP predominantly derived from azurophil granules and that the observed difference in ECP between NO circuits and controls was due to NO enhanced azurophil degranulation from neutrophils and monocytes. However, the observed difference in magnitude of ECP and EPO exocytosis could theoretically also be caused by selective ECP degranulation from eosinophils. Both difference in the adherence to a foreign surface and a variation of quantitative response to the same stimuli in different leukocyte subsets $^{261}$ could cause the reduced degree of degranulation in eosinophils compared to neutrophils. The data thus indicated that when a difference in ECP is detected, it would be advisable to further analyse the material with neutrophil and eosinophil specific markers in order to investigate the relative contribution of each of these granulocyte subsets to inflammatory reaction.

**Basophil degranulation**

Basophil degranulation during clinical ECC is both reported and denied $^{12,13,165-167}$. The 250-fold elevated histamine levels in the controls in the present study clearly demonstrated foreign surface contact during ECC as an
important basophil activator in adults, and brought new light to this controversial issue. The detected histamine levels in controls were in line with previous clinical studies, in which no histamine elevation was observed during clinical ECC 13,164,165,167; whereas, some studies with increased histamine release during ECC 12, 262 report 5-fold higher levels, suggesting an additional contribution in clinical situation from ischemia reperfusion, surgical trauma, perfusion details, anaesthesia or different sampling and analytical procedures. Even small increases in plasma histamine can provoke profound biological effects 163 and the observed levels were above 1 ng/ml and hence considered pathological 13. The observed NO stimulatory effect on basophils is consistent with a previous suggestion, based on clinical observation, that nitroprusside administration may increase histamine release 13, but contradicts nitroprusside as a basophil stabiliser 263. Although the histamine levels increased 250-fold in controls and 700-fold in NO circuits at 80 ppm, the proportion of total histamine released in controls (4%) and NO circuits (11%) was lower than for HNL and MPO. As with eosinophils, lack of basophil adherence to foreign surfaces or variation of quantitative response to the same stimuli in different leukocyte subsets 261 could explain the discrepancy. However, histamine metabolism in the circuit to methylhistamine could theoretically lower accumulated histamine concentrations and the current results may thus underestimate the true degree of basophil degranulation.

Overall, the addition of NO with a foreign surface and as a stimulus for granulocyte activation caused enhanced neutrophil and basophil degranulation; whereas, the effect on eosinophils could not be convincingly demonstrated. Although MPO, HNL, ECP, EPO and histamine increased steadily in controls and NO circuits, there were differences in granulocyte subset adherence to surfaces and proportional and absolute degranulation, indicating that the combination of surface activation and the amount and type of soluble mediators defined the magnitude of granule release in each granulocyte subset. However, it remains to be determined if surface activation or synergy of several parallel mediator stimuli caused neutrophil and basophil responsiveness to NO, or, if NO increased neutrophil and basophil responsiveness to foreign surfaces or mediator stimuli. Furthermore, the intracellular mechanisms associated with a foreign surface and NO activation were not investigated. The NO enhanced neutrophil and basophil degranulation could have significance in several pathophysiological conditions.

5.1.2 ROS producing capacity
The capacity to produce ROS during and after CPB, and production of ROS oxidised proteins during CPB are controversial 57, 74, 76, 77, 78. Values were measured as peak values in Paper I, and as area under curve (AUC) in Paper II, which is biologically a more efficient measurement, also experimental conditions differed. Subsequently, the results from Paper II were more reli-
able. Furthermore, instead of directly measuring ROS levels or ROS effect on proteins in the circuit, both leukocyte and whole blood capacity to release ROS were determined after collection of leukocytes and blood from the circuit and subsequent stimulation with PMA or SOZ. PMA activation of leukocytes, as a receptor mediated activation, is methodologically more efficient than the unspecific SOZ stimulation.

When combining the results from Papers I and II, a contradictory picture was revealed: $O_2^-$ production capacity initially increased after PMA activation in leukocytes and then decreased and decreased after SOZ activation in leukocytes and in whole blood; other ROS production capacity decreased in leukocytes, and increased in whole blood. The data from the present studies are thus inconclusive and could be interpreted to support the previous observation that granulocyte priming may occur by foreign surfaces 79, in addition to pre-CPB priming by heparinisation and surgical wound 27, 264. Alternatively, it could be interpreted that foreign surface contact causes hyporeactivity due to exhaustion or development of tolerance, which is mediated by downregulation by emerging IL-10 production, autoregulation or release of neutrophil degranulation products, such as elastase 265. Absent NO effect on the capacity to release ROS was unexpected, as NO is suggested to act as an ROS scavenger, inhibitor of NADPH oxidase and inducer of glutathione synthesis 266, and is thus theoretically capable of decreasing measured ROS variables. If NO effect on NADPH oxidase was mediated by NO direct mechanisms, and not by nitrosation, nitrosylation or tyrosine nitration, the discrepancy could be explained by the NO half-life being too short to affect ROS production when measurements and stimulation were performed outside the circuit, after sampling and isolation of leukocytes. The 40 and 80 ppm used were low (Papers I and II), compared with the 100-1000 ppm used in platelet studies 248, 267, 268, and higher NO doses may be required to affect ROS release and ROS production. NO could also have reacted with ROS to form peroxynitrate, thus being chemically neutralised and unavailable to exert an effect on NADPH oxidase.

5.1.3 Cytokine release

In general, it is proposed that the cytokines peak several hours after CPB and the main effect is thought to be in the postoperative period 6, whereas interleukin release during SECC is controversial both regarding the time point of elevation and applicability of results to clinical bypass 105. IL-1$\beta$ levels were unchanged in controls and NO circuits and thus pro-inflammatory IL-1$\beta$ could not cause the increased leukocyte granule release. The lack of foreign surface stimulation of IL-1$\beta$ release is similar to previous studies in clinical CPB 115, 37, 116, but contradicts studies in simulated ECMO 110 and clinical cardiac surgery 105, 112-114.
IL-10 increased during recirculation, which concords with previous clinical CPB studies, but is in contrast to one SECC study. Furthermore, IL-10 increased in parallel with a decrease in ROS producing capacity, thus suggesting IL-10-mediated inhibition of ROS producing capacity in response to foreign surfaces. As there was no change in IL-1β, the previously observed NO-mediated decrease in IL-1β release could not be verified.

5.1.4 Cell counts

Decrease in WBC, neutrophil and monocyte count were in agreement with previous SECC studies and could be explained by adherence of cells to the artificial surfaces of the SECC circuit. However, the situation is somewhat different in vivo with initial neutropenia, followed by a return to baseline values and subsequent overshooting to leukocytosis due to cell recruitment from bone marrow. Platelets decreased at 60 minutes in all studies indicating, according to previous SECC and in vivo studies, adherence to the surface. The unchanged eosinophil count in Papers I, II, III contradicted previous experimental dialysis, clinical dialysis, CPB studies, whereas a decrease in eosinophils in Paper IV agreed with previous studies. Subsequently, the eosinophil reaction appeared less consistent than neutrophil reaction, indicating a lesser degree of adhesion or none at all. Furthermore, lymphocyte and basophil counts did not decrease during recirculation and methemoglobin peaked only at 5.6% at the highest NO dose; thus, excluding general toxic NO induced lysis as a cause for decreased WBC, neutrophil and monocyte counts.

The similarity in neutrophil count between NO circuits and controls in Papers II and III and higher neutrophil counts in controls than in NO circuits at the end of Papers I and IV accorded with a previous porcine study. In addition, the lack of difference in platelet counts between NO circuits and controls in Papers I, II, III, IV was in agreement with previous results from ex vivo NO studies, nitro donor and in vivo NO studies. As a whole, the results indicated that NO does not inhibit granulocyte adhesion to the foreign surfaces and that previously observed NO induced decrease in platelet granule release in ex vivo and in animal studies is not associated with preserved platelet counts in the circuit.
5.2 Protein system response

5.2.1 Contact system

The present thesis revealed a new potentially important aspect of the regulation of contact system during inflammatory reactions. In agreement with previous studies with plasma systems, FXIIa-C1INH complexes were predominant at baseline. Furthermore, the activation of FXII during the first hour of recirculation accorded with previous whole blood experiment in SECC. However, in contrast to previous reports, FXIIa-AT complexes increased and FXIIa-C1INH complexes remained stable during recirculation, indicating a predominance of AT-mediated inhibition of FXIIa in whole blood and resembling the condition of plasma in hereditary angioedema. In contrast to previous data, FXIa was primarily complexed with AT instead of C1-INH and remained, after cleavage of minor degree of FXI to FXIa during the first hour, predominantly complexed with AT. This contradicts previous data from plasma and protein systems. Thus, activation of granulocytes by surfaces influenced the contact system balance, indicating that neutrophils play a more active role in the system than previously thought.

There are several possible explanations for AT-complex predominance in both FXIIa and FXIa complex formation in a whole blood milieu. First, although heparin concentration less than 32 U/ml has little influence on AT or C1INH complexing with α-FXIIa and β-FXIIa in plasma milieu, heparin can inhibit C1INH complex formation with β-FXIIa and could theoretically catalyse FXIIa and FXIa complexing with AT in blood milieu. Second, modest hypothermia can inhibit C1INH. Third, complement system and kallikrein consume C1INH. Fourth, FXIIa and FXIa complex formation on granulocyte surface could theoretically prefer C1-INH, leaving more available AT or AT-complexes that are released more easily to plasma from granulocyte surfaces. Fifth, granule contents from activated granulocytes and platelets can participate in complex equilibrium of FXII and FXI activation and inactivation. Proteases in plasma or on neutrophil surface can cleave, and thus inactivate, C1INH. In addition, FXIIa production in whole blood in the current study and lack of β-FXIIa production in plasma milieu, indicate direct FXII activation by released proteases, sulphated proteoglycans or proteases bound to granulocyte plasma membrane. A caveat for the protease effect is the lack of difference in contact activation between the groups, despite differences in neutrophil and basophil degranulation contents in plasma which indicate an absent dose response effect in observed concentrations, or FXII and FXI interaction with eosinophil-specific granule-contents.
5.2.2 Complement system

A previous study suggests initial fast complement activation during the first five minutes of recirculation \(^{14}\). In the present study, the first observation time was at 60 minutes, where after the steady increase at detected later time points was similar to previous SECC studies \(^{14, 276}\). In contrast to previous inhibition of complement activation by nitroprusside both in heparin-coated SECC \(^{255}\) and in one clinical ECC study \(^{277}\) and increase of complement activation in one clinical study with nitroglycerin \(^{278}\), NO did not affect complement activation. Furthermore, despite a difference in degranulation protein levels, the similar complement product levels between NO circuits and controls indicated that granule contents released to extracellular milieu did not affect complement system activation within the observed concentrations.

5.2.3 Coagulation

In contrast to a previous clinical study \(^{279}\) and present studies (Papers II and III), active clotting time (ACT) decreased in NO circuits in Paper IV. In Papers II and III, 10 U/ml heparin was combined with lower doses of NO, 40 ppm or 80 ppm, whereas in Paper IV, 500 ppm NO was used with 4.4 U/ml heparin. Furthermore, a non-parametric Kruskal-Wallis test measuring medians was used in the latter study because of variance within the NO group, but revealed no difference between the groups, although application of parametric ANOVA would have resulted in a significant difference between the groups. The data thus indicated that NO may result in a shortening of ACT time in some patients. Several mechanisms of actions at molecular and cell levels are possible. First, NO can directly neutralise heparin \(^{280}\), or, reduce thrombin sensitivity for heparin-AT complex \(^{281}\), or via tyrosine nitration enhance fibrinogen coagulation properties \(^{140}\). Second, MPO \(^{242}\) and eosinophil granule contents \(^{158}\) can inhibit heparin, and release of elastase or cathepsin G from azurophil granules can activate coagulation \(^{243}\). Third, formation of monocyte-platelet and PMN-platelet aggregates with ongoing serine protease release can cause TF release from platelets \(^{230}\). Fourth, activated monocytes can produce TF \(^{2}\). However, platelet factor-4 mediated inactivation of heparin effect is not probable as NO inhibits platelet degranulation.

There are data suggesting that the interaction between heparin and NO, or heparin and nitrodonors, is clinically relevant in cardiology, interventional radiology and in cardiac surgery. The use of parallel nitroglycerin and heparin attenuates the anticoagulant effect of heparin in acute coronary syndromes in cardiology, interventional coronary procedures \(^{282}\) and cardiac surgery \(^{283}\) by shortening APTT \(^{282, 283}\). However, NO net effect on coagulation in a heparinised patient in vivo is complicated to predict due to the presence of various pro- and anti-coagulative substances released from an array.
of cell types associated with specific conditions. This is exemplified by NO being referred to as an anti-thrombotic agent in several SECC and animal models because of its inhibitory effect on platelet activation and platelet granule release, as well as through an anti-thrombotic effect on monocytes, endothelium, smooth muscle cells and coagulation in ex vivo and animal models. However, neither 40 ppm nor 100 ppm has an effect on the platelet count or platelet exocytosis of β-TG in clinical CPB, whereas 80 ppm decreases clot formation in oxygenator in CPB.

In conclusion, the shortened ACT time in some circuits (Paper IV) indicated that high iNOS activity in pathophysiological states in tissues, or the administration of high doses of exogenous NO gas, or possibly NO donor, in combination with activated leukocytes and platelets could result in intravascular coagulation, despite heparinisation. This limits the use of higher doses of NO or NO donor parallel with heparin, or warrants more aggressive monitoring of coagulation during CPB, until the effects of nitroglycerine and nitroprusside are fully clarified in clinical CPB.

5.3 iNOS and NO metabolites

L-NAME is a widely used non-specific cNOS and iNOS inhibitor with preference for cNOS. In line with a previous study with L-NMMA, L-NAME did not affect degranulation (Paper IV), suggesting that granulocyte cNOS or iNOS are not involved in neutrophil degranulation in SECC. NO is suggested to act in biphasic way in leukocyte degranulation: low doses of NO inhibit degranulation, whereas, higher doses overcome the inhibition and stimulate exocytosis. Nevertheless, the contrary has been suggested. L-NAME can theoretically neutralise the previously reported NO stimulatory and inhibitory effect on degranulation by inhibiting both cNOS and iNOS. Furthermore, short incubation times, and high initial intracellular l-arginine concentrations can inhibit L-NAME uptake and thus inhibit its biological effect. The absent iNOS mRNA expression in leukocytes (Paper IV), the absent increase in total NO$_2^-$/NO$_3^-$ in controls (Papers II, III) and the lack of L-NAME effect on both NO$_2^-$ and NO$_3^-$ generation (Paper IV) corresponded with previously undetected iNOS mRNA in neutrophils and stable NO$_2^-$/NO$_3^-$ in clinical cardiac surgery. In contrast, the presence of iNOS mRNA is reported in neutrophils and eosinophils and the existence of unchanged iNOS mRNA levels are suggested in isolated leukocytes before and after cardiac surgery, and NO$_2^-$/NO$_3^-$ are reported to be generated. The discrepancy in findings indicates that foreign surface activation alone may not be sufficient, but that additional stimuli, such as bacteria and endotoxin, multiple cytokines or ischemia are required in order to activate two or more signal transduction pathways to induce or increase iNOS mRNA
production. Furthermore, IL-10 produced during ECC (Paper II) can theoretically inhibit iNOS mRNA expression. Subsequently, the observed discrepancy in NO$_2$/$\text{NO}_3^-$ production in clinical CPB studies may be explained by granulocyte capacity to produce NO$_2^-$ independent of NOS, or NO production by iNOS in tissues or granulocytes due to endotoxaemia, surgical trauma or ischemia-reperfusion and not due to foreign surface activation.
6. Conclusions

- NO addition increases surface activated neutrophil and basophil degranulation, but does not affect eosinophil granule release

- NO addition does not affect leukocyte ROS producing capacity or cytokine production in SECC

- NO addition does not affect foreign surface activation of either contact or complement systems

- In the presence of activated leukocytes, FXIIa and FXIa are predominantly inactivated by AT in whole blood

- NO-inhibitor L-NAME has no effect on neutrophil degranulation and nitrite/nitrate production

- iNOS is not expressed in surface activated leukocytes
7. Future aspects

Results from this study will probably have wider applications in other inflammatory diseases than postperfusion syndrome related to extracorporeal circulation. iNOS is upregulated and NO produced in transplant rejection, sepsis, and rheumatoid arthritis as well as in localised inflammatory states, whereas MPO is a predictor of death in myocardial infarction and has potential role in development of atherosclerosis. ECP is associated with asthma. The concept of NO as an enhancer of activated neutrophil degranulation is appealing and provides a great amount of research opportunities in future. Further studies are required to verify the NO enhanced degranulation in basophils and neutrophils in cell and animal models and evaluate, if the results are applicable in human pathological and clinical conditions. Furthermore, the mechanism of action for NO was not object of this thesis and needs to be clarified. This could provide opportunities for novel therapeutic strategies in inflammatory diseases.


Kväveoxid (NO) är en gas som produceras i kroppen av flertalet celltyper och dess produktion ökar i t.ex. asthma, blodförgiftning och reumatoid artrit. NO kan även användas som medicinsk gas i svårt sjuka respiratorbehandlade patienter, ges som nitroglycerin till hjärtsjuka patienter och indrages i höga koncentrationer till lungor i cigarettrök. NO reglerar och är involverad i flertalet viktiga processer i kroppen såsom hålla blodkärl öppna, vidga luftvägar samt reglera livmoder under graviditeten.

Denna avhandling har studerat hur NO påverkar de olika cellerna och äggvitämnen i inflammatoriska reaktionen. Blod från frivilliga donatorerna cirkulerades i hjärt-lungmaskin och den inflammatoriska reaktionen mot främmande ytan i hjärt-lungmaskin och NO påverkan på denna studerades. Resultaten visade att NO ökade utsöndring av innehållet från två olika typer av vita blodkroppar men påverkade inte äggvitesystemen, som aktiveras av kontakten mellan blodet och de främmande ytorna. Ansamlingen av vita blodkroppar till vävnaderna och frisättningen av deras innehåll förgörer vävnadsskador och inflammation. Alltsammans tyder resultaten på att NO utövar en inflammationsbromsande effekt och resultaten från denna avhandling kan användas för utvecklandet av nya metoder för att minska inflammatoriska reaktionen i hjärtsjukdom och övriga inflammatoriska tillstånd.
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