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Critical evaluation of nitric oxide as an immuno-modulator in humans

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ABSTRACT

Introduction: Nitric oxide (NO) has been shown to possess anti-inflammatory properties. In a porcine endotoxin model the combination of intravenously (iv) administrated glucocorticoid (gc) and inhaled NO (iNO) compared to gc or iNO given separately, blunted the inflammatory response in vital organs. The ischemia and reperfusion (I/R) syndrome has an essential role regarding the pathogenesis in a number of clinical conditions. Animal and human studies have shown favourable effects with exogenously administrated NO in different I/R models.

Aim: The overall aim of this thesis was to study the potential of inhaled NO as a modulator of the inflammatory response in humans. In study I-III two different concentration of iNO in combination with gc was studied and evaluated using clinical parameters and cytokines in plasma (study I-II) as well as microparticles in plasma (study III). In study IV we evaluated whether knee surgery in spinal anesthesia during tourniquet could be used as a model to study I/R, and if so, if this could be attenuated by iNO.

Methods: In study I-III an endotoxin model was employed in 30 healthy human volunteers participating in 60 experiments. Study III is based on samples taken separately in study II. The studies were double-blind, cross-over and randomised with regard to iNO and placebo (nitrogen, N_2), i.e. every volunteer had iv endotoxin (2 ng/kg) and iv gc (2 mg/kg) combined with iNO in one experiment (iNO/gc) and with placebo (placebo/gc) in the other. In study I endotoxin was given before gc and iNO (30 ppm lasting for 5 hours), while in study II iNO (80 ppm, lasting for 7.5 hours) was initiated first, followed by endotoxin and gc. Clinical symptoms were recorded and blood samples collected. In study IV patients consecutively submitted to knee arthroplasty in spinal anaesthesia were included. As a standard procedure a tourniquet was used to create a bloodless surgical field. The patients were randomised into three groups (n=15). Groups 1 and 3 were either receiving iNO 80 ppm or placebo throughout the entire operation, whereas group 2 received iNO 80 ppm just in the beginning and in the end of the operation, hence, no iNO during the period with activated tourniquet. Blood samples and muscle biopsies were collected during the operation. Adhesion molecules before and after the ischemic period were analysed.

Results: In study I and II endotoxin elicited typical flulike symptoms e.g. headache and fever as well as activation of cytokine levels. In study III there was an increase in platelet and monocyte microparticles (MP) while endothelial derived MP were unaltered. Also, platelet derived MP positive to CD40L and monocyte MP positive to HMGB1 showed an early increase. There was no difference between the two treatments (iNO/gc and placebo/gc) in study I-III. In study IV no signs of endothelial cell activation or inflammatory response neither systemically nor locally in adjacent muscle were seen.

Conclusions: The present human endotoxaemic model exhibited reproducible results, thereby providing a stable and safe model for randomized studies. The combination of intravenously administered gc and iNO, elicited no anti-inflammatory effect. The endotoxin infusion in healthy volunteers resulted in an increase in plasma cytokines as well as in microparticles released from platelets and from monocytes but not from the endothelium. In patients undergoing knee arthroplasty in spinal anesthesia, the ischemia/reperfusion created by a tourniquet did not cause any signs of endothelial cell activation or inflammatory response.

LIST OF PUBLICATIONS

I. Nitric oxide inhalation and glucocorticoids as combined treatment in human experimental endotoxemia.

Hållström L, Berghäll E, Frostell C, Sollevi A, Soop AL.
Crit Care Med. 2008 Nov;336(11):3043-7

II. Immunomodulation by a combination of nitric oxide and glucocorticoids in a human endotoxin model.

Hållström L, Berghäll E, Frostell C, Sollevi A, Soop AL
Acta Anaesthesiol Scand. 2011 Jan;55(1):20-7

III. HMGB1 is exposed on circulating monocyte microparticles following endotoxin challenge in human volunteers.

Soop A, **Hållström L**, Frostell C, Wallén H, Mobarrez F.
Manuscript

IV. Tourniquet exsanguination/ischemia during knee surgery and spinal anaesthesia is not associated with signs of inflammation. A randomized placebo controlled study involving inhaled nitric oxide.

Hållström L, Frostell C, Sollevi A, Herrlin A, Lindroos E, Lundberg IE., Soop AL.
Submitted

CONTENTS

LIST OF ABBREVIATIONS

AGE	advanced glycation end products
AP-1	activator protein 1
BW	body weight
CD	cluster of differentiation
DAMP	damage or death-associated molecular pattern
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EMP	endothelial microparticles
Gc	glucocorticoids
GMP	guanosine monophosphate
GR	glucocorticoid receptor
GTP	guanosine triphosphate
HMGB1	high mobility group box protein 1
I/R	ischemia/reperfusion
ICAM	intercellular adhesion molecules
IL	interleukin
LBP	lipopolysaccharide-binding protein
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinases
MP	microparticle
NF- κB	nuclear factor-kappa B
NO	nitric oxide
NOS	nitric oxide synthases
PAMP	pathogen-associated molecular patterns
PMP	platelet microparticles
PS	phosphatidylserine
RAGE	receptor of advanced glycation end products
ROS	reactive oxygen species
SARS	severe acute respiratory syndrome
SIRS	systemic inflammatory response syndrome
TLR 4	Toll-like receptor 4
TNFα	tumor necrosis factor alpha
VCAM	vascular adhesion molecules

INTRODUCTION

INFLAMMATION

The inflammatory reaction involves a response that is the coordinated reaction of the immune system; a collection of cells, tissues and molecules mediating resistance to infections. Inflammation is initiated by a plurality of elements including infections with pathogens or their products (e.g. endotoxins/lipopolysaccharides), tissue damage with following cell injury, as well as episodes with reperfusion after ischemic events. At the site of injury, the inflammatory response causes activation and accumulation of leukocytes and plasma proteins. The inflammation per se may cause tissue damage (1).

THE IMMUNE SYSTEM

Protection of the host is the primary physiological role of the immune system. The immune system is classically divided into the innate immunity that mediates the initial inflammatory response and the adaptive immunity which is the more specific and powerful response system and also a more slowly activated part of the immune system (1).

The innate immunity is always present and prepared to rapidly eliminate microbes. It consists of phagocytes (macrophages and neutrophils that ingest and destroy microbes), natural killer (NK) cells that recognize and kill infected cells, and also dendritic cells that together with macrophages serve as antigen-presenting cells which stimulate the subsequent adaptive immunity. In the innate immune system all the cells of a particular type, e.g. macrophages, have identical receptors on their surface. Hence, many cells are able to recognise the same microbe (1).

Adaptive immune responses develop later in life compared to the innate immunity system. The adaptive immunity is based on antigen-specific cells in contrast to the innate immunity. The adaptive immunity is in itself divided in two groups; humoral and cell-mediated immunity. Humoral immunity is mediated via B lymphocytes producing and secreting antibodies in order to neutralise and eliminate microbes and microbial toxins present outside the host cells. The cell-mediated immunity is represented by T lymphocytes defending against microbes that living and dividing inside the infected cells, thereby inaccessible to antibodies, T lymphocytes recognise antigen fragments displayed on the surface of the infected cells.

The adaptive immunity response often enhances the antimicrobial mechanism of the innate immunity, e.g. by antibodies binding to microbes thereby activating phagocytes, and T lymphocytes (helper T cells) producing cytokines that activate phagocytes (as well as T- and B- lymphocytes). Another feature of the adaptive immune system is the immunological memory protecting the host from being reinfected by the same microbe (1).

EARLY INFLAMMATORY RESPONSE

Phagocytes (neutrophils, monocytes) are among the first cells responding to an inflammatory stimulus, infection or trauma. Phagocytes are recruited from the bloodstream to the site of the initial inflammatory insult in response to chemotactic stimuli (i.e. chemokines, chemoattractant cytokines e.g. IL-8) (2). Monocytes mature into macrophages as they enter non-vascular tissue. At the site of the insult tissue macrophages and dendritic cells produce cytokines (e.g. TNF and IL-1 and chemokines) that activate the endothelium. This activation leads to the production of adhesion molecules and cytokines, the latter causing further activation of leukocytes and monocytes. The neutrophils and monocytes have to migrate through the vessel wall in order to reach the site of inflammation, this requires adhesion molecules expressed on the activated endothelium and leukocytes/monocytes.

Neutrophils and macrophages recruited to the site of infection ingest and destroy microbes by means of nitric oxide (NO, produced by inducible nitric oxide synthetase, iNOS), reactive oxygen species (ROS) and enzymes, these products may be liberated extracellulary and thereby contribute to the inflammatory reaction and tissue damage (1, 3, 4).

INTERACTION BETWEEN ENDOTHELIAL CELLS AND LEUKOCYTES

Adhesion molecules are of great importance in the inflammatory host response as they mediate the interaction between leukocytes and endothelial cells. The interaction involves three stages; rolling (low affinity interaction), firm adhesion and transendothelial migration (3) (Fig. 1).

During the leukocyte rolling the adhesion molecules E- and P-selectin on the

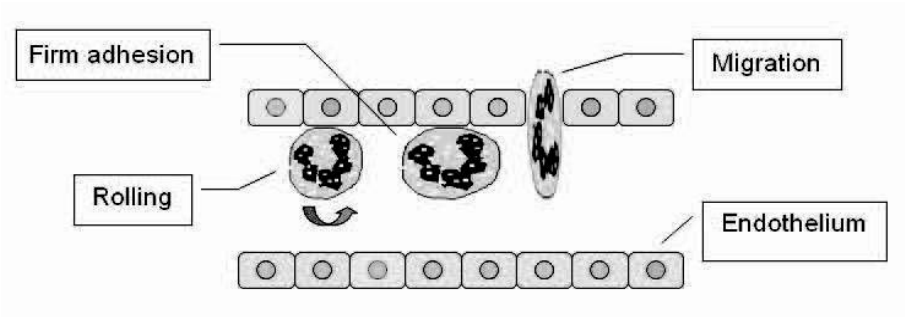

Figure 1. Three stages of leukocytes interaction with the endothelium in response to activation.

endothelium interact with corresponding ligands on leukocytes (e.g L-selectin). Integrins (CD11a/CD18 and CD11b/CD18) are glycoproteins expressed on the surface of leukocytes, function as ligands interacting with ICAM (intercellular adhesion molecule) and VCAM (vascular adhesion molecule) expressed on the endothelium to enable firm adhesion, and finally the interaction between PECAM-1 (platelet-

endothelial cell adhesion molecule) expressed both on the endothelium and on leukocytes enable the migration through the vessel wall (3, 5, 6). The major site of adhesion (rolling) is the postcapillary venule where the interaction between leukocytes and endothelium occur, all dependent on adhesion molecules (3). Monocytes and activated T lymphocytes are believed to follow the same mechanisms in order to reach the site of infection/inflammation (1, 3).

ICAM and VCAM are constitutively expressed on the endothelial cell surface, and may, after inflammatory stimuli, be further induced leading to enhanced amounts (7, 8). P-selectin is found in storage granules (Weibel-Palade bodies) in the endothelia cells as well as in platelets (alpha granules) and is rapidly mobilized, within minutes, to the cell surface upon inflammatory stimulation (5). E-selectin expression is under transcriptional control and may be induced by cytokines such as TNF and IL-1 and is therefore not as rapidly expressed as P-selectin in response to inflammatory stimulation (3). Soluble forms of adhesion molecules are detectable in biological fluids and their concentrations may reflect an increased expression on cellular level due to an inflammatory response (6, 9).

CYTOKINES

The first proposal that soluble factors may influence inflammatory reactions was made in 1944 when Menkin "purified" fever generating properties from inflammatory exudates and named these factors "pyrexins". In 1953 Bennett and Beeson were able to show that in acute inflammatory exudates endogenous pyrogens could be separated from bacterial pyrogens (endotoxins). They also managed to extract endogenous pyrogens from leukocytes in peripheral blood. In 1974 the term "cytokines" was proposed for the family of proteins produced and secreted by different cell types promoting the immunologically mediated inflammatory reactions. Today most cytokines are called interleukins (IL) because they are molecules produced by leukocytes and acting on leukocytes. However, this is a far too limited definition since many cytokines/interleukins are produced by, and act on, other cells than leukocytes (1, 10).

Cytokines are soluble proteins functioning as molecular messengers between cells, they interact with the immune system in order to regulate various inflammatory responses as well as having an impact on cell growth, differentiation and development (10). The cytokines interact with target cells via high-affinity receptors, these receptors may be located on the cell producing the cytokine (autocrine action), on adjacent cells (paracrine action) or even distributed to distant target cells (endocrine action) (1, 10, 11).

In the immune system many cytokines promote the inflammatory reactions, so called pro-inflammatory. There are also cytokines that function mainly as down-regulators of the inflammatory response, e.g. transforming growth factor B (TGFβ) and IL-10. Some microbes have "taken advantage" of the down-regulating mechanisms, an example

being the Epstein Barr Virus (EBV) producing a "virokine" version of IL-10, resulting in a suppression of the host resistance (10).
Macrophages and dendritic cells are the major origin of cytokines in the innate immune system while T cells are the main source in the adaptive immune system (1).

HIGH MOBILITY GROUP BOX PROTEIN 1 (HMGB1) AND ITS RECEPTORS

HMGB1

Previously, HMGB1 was known as a protein existing in profuse amounts in the cytoplasm and in the nucleus of most cells, being a nuclear component interacting with DNA to modify its structure and regulate gene expression (12). It is a highly conserved protein with a 99% identical amino acid sequence homology in mammals (13). In 1999 HMGB1 was revealed as an important late mediator of sepsis in an endotoxin model on mice. It was shown that antibodies to HMGB1 decreased endotoxin induced lethality and that administration of HMGB1 in itself was lethal. Also, in septic patients, an elevated level of HMGB1 in serum was correlated with outcome (survivors/non-survivors) (14).
In response to proinflammatory stimuli (e.g. LPS, TNFα and IL-1β) HMGB1 translocates to the cytoplasm and is released extracellulary by activated monocytes/macrophages, thus acting as a cytokine (15). It has been shown that HMGB1 is passively released from necrotic or damaged, but not apoptotic cells, due to the tight bonding of HMGB1 to chromatin (the combination of DNA and proteins in the nucleus) (13, 16). However, in recent years, observations have indicated a relationship between apoptosis and the release of HMGB1 (17, 18). Dying/apoptotic cells produce ROS in mitochondria leading to the oxidation of HMGB1. The importance of the HMGB1 redox status as a generator of inflammation cannot be underestimated since it has been elicited to inhibit the inflammatory response (19). On the other hand, the reduced form of HMGB1 activates inflammatory cells (19, 20).
Due to its immunological properties HMGB1 released from damaged tissue can be designated as an alarmin or DAMP (damage or death-associated molecular pattern), a signal of traumatic cell death (13, 18). The release of HMGB1 promotes signals that lead to both a pro-inflammatory response as well as tissue regeneration (13, 18). The passive release of HMGB1 from necrotic/damaged cells is believed to occur early, in contrast to the active secretion that occurs later. Consequently, HMGB1 has been designated to serve as a late mediator of sepsis, but as an early mediator of tissue damage e.g. in ischemia reperfusion (I/R) injury (12, 14, 21). In mice, an increased level of HMGB1 has been seen 8-32 hours after endotoxin challenge and 18 hours after surgical induction of peritonitis (14, 22).
It has been shown that HMGB1 is of vital importance in mediating injury in I/R. HMGB1 protein expression in the liver was up-regulated one hour after reperfusion and in cerebral ischemia 4 hours after start of the ischemic event, also, after 30 minutes in the heart, all in murine experiments (21, 23, 24). In patient studies with cerebral and

myocardial ischemia respectively, elevated levels of HMGB1 were seen within 24 hours (24, 25). HMGB1 has been demonstrated to interact mainly through receptor of advanced glycation end products (RAGE) (23, 24) and Toll-like receptor 4 (TLR4) (21, 26) but also via other receptors in the TLR family (27).
Apart from inducing cytokine secretion HMGB1 facilitates the inflammatory response by activating the endothelial production of adhesion molecules, also, interaction with RAGE results in migration of monocytes (28, 29).

Receptor of advanced glycation end products (RAGE)

RAGE is a transmembrane multiligand receptor, binding to a variety of ligands such as HMGB1 and advanced glycation end products (AGE) (30). AGE are macromolecules such as proteins, lipids and nucleic acids formed by glycation. The production of AGE is higher in pro-oxidant environment and in diabetics due to the altered glucose metabolism (31). The interaction of RAGE with HMGB1 mediates cytokine production in monocytes and neutrophils by activating several intracellular signal pathways including the nuclear factor-ĸB (NF- ĸB) and mitogen-activated protein kinases (MAPK) (30, 32).
Soluble RAGE (sRAGE), a truncated non cell bound isoform of RAGE circulates in plasma functioning as a "decoy" and may thereby prevent activation of the cell surface receptor (30). The levels of sRAGE have been shown to be elevated and associated with outcome in septic patients (33). In patients with higher baseline sRAGE an association with increased severity of acute lung injury and increased mortality has been seen (34). Also, in a model with human volunteers given a moderate dose of endotoxin a significant increase in sRAGE was demonstrated (35).

ENDOTOXIN CHALLENGE AS A MODEL FOR INFLAMMATION

Sepsis and the systemic inflammatory response syndrome (SIRS) is a result of failure in the host infection control mechanisms and is often initiated by bacterial lipopolysaccharide (LPS/endotoxin) (36). In USA more than 750 000 cases of severe sepsis are estimated to occur every year with an in-hospital mortality of 29 to 50% or even higher if the patients develop septic shock (hypotension not responding to fluid resuscitation) (37). The mechanism underlying endotoxin shock was described by Hinshaw in 1956-58, but the role of bacterial toxins was described as early as 1888 by Roux and Yersin (36).
Endotoxin / bacterial lipopolysaccharide (LPS) is found in the outer membrane of various Gram-negative bacteria. It consists of a hydrophilic polysaccharide (sugar) chain on the surface of the bacterial cells and a hydrophobic lipid moiety known as lipid A that is located in the outer leaflet of the outer cell membrane, the latter responsible for the toxic effects. The LPS structure may differ between different bacteria. The shifts in structures are not necessary for the survival of the bacteria, but have implications regarding its virulence (38).

LPS is one of the most forceful pro-inflammatory agonist for human leukocytes (39). LPS interacts with TLR4, facilitated by CD14 and lipopolysaccharide-binding protein (LBP) (38). This interaction activates nuclear factor kappa B (NF-ĸB), a so called stress-responsive transcription factor that regulates the gene expression of different cytokines. The activation results in the synthesis and release of various cytokines considered characteristic in sepsis and the systemic inflammatory response syndrome (SIRS). NF-ĸB may also be induced by cytokines through their respective receptors on the cell membrane (4).

The use of endotoxin challenge in human volunteers in order to induce a systemic inflammatory reaction has been proved to be a safe, reliable and reproducible method (40). The model has been used for several years in order to study physiological reactions such as cardiovascular parameters and cytokine release, but also potential anti-inflammatory effects of different drugs like glucocorticoids, ibuprofen and adenosine (41-44).

EXOTOXINS

These proteins can be produced inside both gram-positive and gram-negative bacteria. Once outside the bacteria the exotoxin may function as cytolytic enzymes causing membrane disruption, or as receptor binding proteins that alter the function of or kill the cell.

Due to the receptor binding, many exotoxins from pathogens such as Vibrio cholera or Clostridium botulinum are highly specific in targeting tissues in contrast to endotoxin (45).

GLUCOCORTICOIDS

Glucocorticoids are a class of steroid hormones built from the lipophilic cholesterol skeleton. Hence, they penetrate easily the cell membrane and reach all tissues including the brain (46). The glucocorticoid receptor (GR) complex is found in the cytoplasm of cells. After interaction between glucocorticoid and GR the complex is translocated into the nucleus where the GR complex interacts with target genes and transcriptions factors such as NF-ĸB, in order to enhance or repress transcription. The major anti-inflammatory and immunosuppressive effect of glucocorticoids is considered to be the inhibition of pro-inflammatory cytokines and the increase of anti-inflammatory protein production like IL-1ra (47, 48).

NITRIC OXIDE

In 1980 endothelium dependent relaxation was first described by Furchgott and Zawadzki, who found that the relaxation on isolated blood vessels by acetylcholine required the presence of endothelial cells (49). Further studies revealed that stimulated endothelial cells did release a factor that diffused to the smooth muscle cells where it caused relaxation, endothelial-derived relaxing factor (50). This substance with vasodilatation properties was in 1987 proposed to be nitric oxide (NO) (51). NO

consists of one nitrogen atom covalently bound to an oxygen atom with one unpaired/free electron, this makes the molecule a highly reactive free radical (52).
Continued studies have revealed NO as a molecule with many important features in addition to vasoregulation. NO has been shown to possess anti-inflammatory properties by limiting platelet activation/aggregation as well as leukocyte adhesion to the endothelium (53, 54). Also, NO reduces the production of proinflammatory cytokines in the human endothelium (53), macrophages (55) and neutrophils (56) as well as regulating (reduction) myocardial contractility (54). Further, NO has been revealed to function as a signalling molecule in both the central and the peripheral nervous system, and when produced by macrophages and neutrophils, a defending mechanism against microbes (54, 57). In 1992 NO was declared "molecule of the year" by Science Magazine due to its properties as an important messenger molecule and part of the immune defence (58). Robert Furchgott, Louis Ignarro and Ferid Murad received the Nobel Prize in 1998 for their discoveries concerning "the nitric oxide as a signalling molecule in the cardiovascular system" (www.nobelprize.org) (51, 58).
Endogenous NO is synthesized by a family of NO synthases (NOS) catalysing the conversion of L-arginine to the free radical NO and L-citrulline. There are three isoforms of NO synthase, initially named after the tissues they first were identified in; neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). The latter is expressed in several cell types including macrophages and neutrophils in response to inflammatory stimulus (51, 59). From endothelial cells NO diffuses, catalyzed by eNOS, to the smooth muscle cells as it is highly lipid soluble and membrane permeable (60). In the smooth muscle cells NO activates soluble guanylate cyclase causing vasodilatation via the synthesis of cyclic GMP from GTP (51, 61).
When the combination of iNO and intravenous glucocorticoids was used as a treatment after endotoxin challenge in a porcine model lasting for 6 hours, the inflammatory response in systemic organs was blunted compared to treatment with iNO or glucocorticoids separately (62). In this porcine model endotoxin infusion decreased glucocorticoid receptor (GR) expression in lung, liver and kidney tissue. iNO was considered to induce an upregulation in GR expression and it was suggested that this GR upregulation made glucocorticoids more effective (62).

NITRIC OXIDE AND ISCHEMIA REPERFUSION INJURY

The ischemia and reperfusion (I/R) syndrome has an essential role regarding the pathogenesis in a number of clinical conditions such as stroke, myocardial infarction and organ transplantation (63-65). Reperfusion, necessary in providing the post-ischemic tissue with oxygen and other metabolic substrates after ischemia results in acceleration of the cellular necrotic process and may cause the same consequences as prolonged ischemia (63, 66). The formation of reactive oxygen species (ROS, e.g. superoxide, O_2^-, or hydrogen peroxide, H_2O_2) contributes to the expression of several different adhesion molecules on leukocytes and vascular endothelia in combination with reduced amounts of the antiadhesive NO (66, 67). As a result, neutrophils

emigrate to the extravascular compartment causing microvascular barrier disruption and edema (66, 68). ROS also stimulates cytokine and chemokine production through NF-ĸB activation (69). NO has been demonstrated to affect the adherence of leukocytes and platelets to the endothelium by downregulating the expression of the adhesion molecules (ICAM, VCAM, P-selectin) (53, 67, 70, 71). The leukocyte adherence to the endothelium results in capillary plugging and edema formation which causes the "no-reflow" phenomenon characterising the I/R injury (72). The balance between ROS and NO seems to be an important factor regarding the leukocyte and platelet adhesion induced by I/R. During oxidative stress, (imbalance between production of oxidizing species and capability of antioxidant defence) as in the case of I/R, eNOS may generate superoxide (O_2^-) (73, 74). Together O_2^- and NO form the highly reactive free radical peroxynitrite ($OONO^-$), a potent inducer of cell death and a substance that has been suggested contributing to the I/R injury (73, 75). However, there are facts indicating that the interaction between NO and O_2^- creating $OONO^-$ could be beneficial since $OONO^-$ possibly acts as a O_2^- radical scavenger. The ratio O_2^-/NO would therefore be of importance during I/R in terms of antioxidation (76).
Other suggested mechanisms for NO in protecting organs against I/R injury are illustrated in Fig. 2 and include; reduction of pro-inflammatory cytokines (e.g. TNF-α and IL-1β) and enhanced levels of anti-inflammatory cytokines (e.g. IL-10) (53, 77),

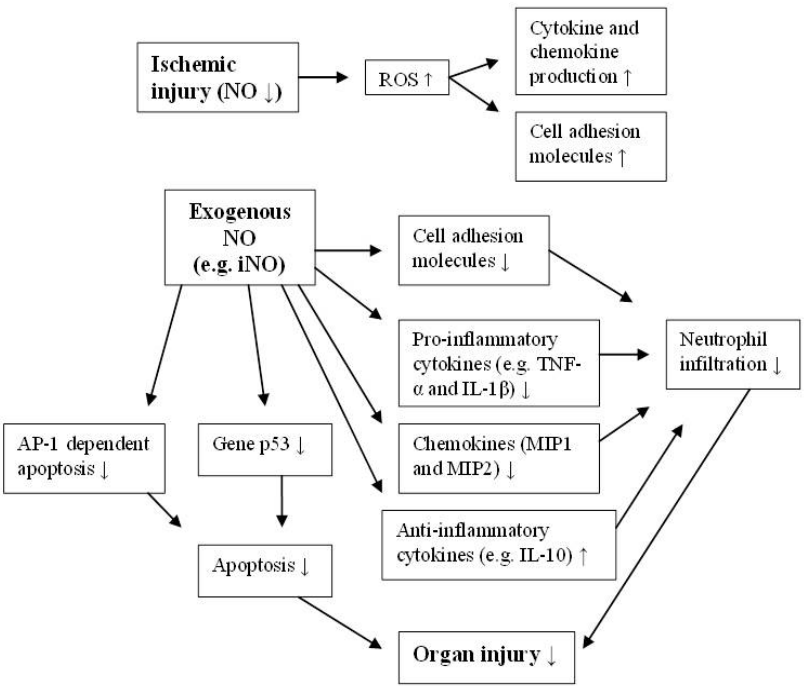

Figure 2. A summary of some possible mechanistic effects by exogenous NO during I/R. Decreased apoptosis and neutrophil infiltration help to protect the cell during ischemia and reperfusion and contribute to diminished organ injury.

MIP-2) (78), inhibit NF-ΚΒ activation (79) and activator protein 1 (AP-1) dependent apoptosis (programmed cell death) (80), inhibit apoptosis by downregulation of gene p53 (81).

The half-life of NO in whole blood is very short (< 2 ms) due to the fast oxidation of oxygenated haemoglobin where methaemoglobin and nitrate (NO_3^-) are produced, and the oxidation of NO in plasma where nitrite (NO_2^-) is formed (Fig. 3) (82, 83). Thus, nitrate and nitrite are oxidative breakdown products of NO long considered as inert

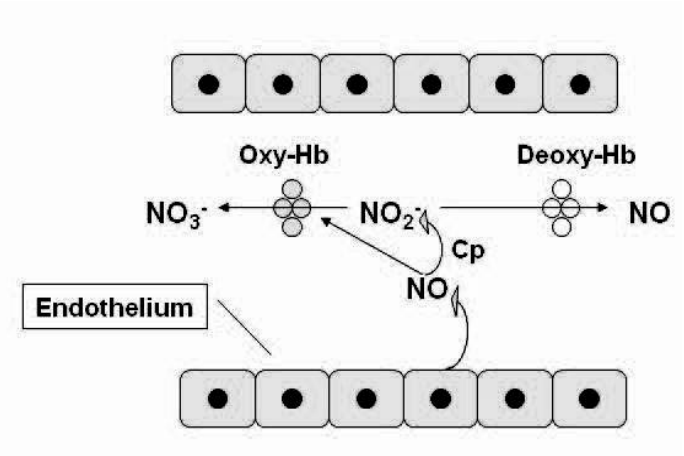

Figure 3. NO synthesized by eNOS in the endothelial cells is in the bloodstream partly converted by plasma ceruloplasmin (Cp) to NO_2^- which serves as a stable reservoir, later capable of reacting with oxy-Hb or deoxy-Hb generating NO_3^- or NO. Cp = ceruloplasmin, NO_3^- = nitrate, NO_2^- = nitrite.

metabolites with limited intrinsic biological activity (84).
Furthermore, nitrite can be reduced to NO by deoxy-haemoglobin where methaemoglobin once again is created. The balance of oxy-haemoglobin/deoxy-haemoglobin affects the vascular tone and blood flow by inactivation or production of NO (83). However, there are also other reductases that can generate NO from nitrite under anaerobic conditions like deoxymyoglobin (85) and xanthine oxidase (86).
Hence, NO produced by nitric oxide synthases (NOS) during normoxia serves as an "ischemic NO buffer" being stored mostly in erythrocytes as nitrite (87), then regenerating to NO during hypoxia and ischemia when the NOS function is limited due to lack of the substrate oxygen (88, 89) and decreased eNOS protein (59). This down-regulation or altered function of endothelial/constitutive NOS (eNOS) seems to be of great significance for the cytoprotection in the I/R scenery (72, 74).
NO administered exogenously (inhaled) has been shown to exert anti-inflammatory properties in I/R settings, both in animal (90-92) and in human (93, 94) studies. In patients, the inflammatory response was attenuated by iNO in a setting where the patients, in general anaesthesia, had knee surgery with a bloodless technique using a

tourniquet (93). In analogy with these findings, an endocrine role of NO has been demonstrated since endogenously derived NO is transported in the blood and metabolised into remote organs, where it mediates cytoprotection in an animal setting of I/R injury (95).

CELLULAR MICROPARTICLES (MP)

MPs were called "platelet-dust" promoting coagulation when first described in 1967 (96). MPs are fragments shed from the plasma membrane of different cell types such as leukocytes, platelets and endothelial cells during activation or apoptosis (97, 98). They are identified using flow cytometry. Approximately 70-90% of microparticles in the blood stream emanate from platelets (99). MPs have different sizes (0.1-1 μm) and composition in proteins and lipids, they also carry different membrane antigens and procoagulant entities such as tissue factor depending on the cell origin (97, 100, 101). MPs are prothrombotic since they have an over-representation of negatively charged phosphatidylserine (PS) on the outer surface, effectively binding coagulation factors (102).

Since MPs carry antigens and receptors from their cell of origin, they can transfer these surface signalling molecules to other cells of different origin, initiating intracellular pathways (102, 103). As well as having pro-coagulant properties, MPs may also directly affect endothelial and monocytic functions contributing to the inflammatory process (104). The suspected involvement of MPs in clinical disease was demonstrated for the first time in 1989 in patients having the rare congenital bleeding disorder called Scott syndrome, it was found that platelets were impaired in their capacity to produce MPs (105). Subsequently, in 1992 it was demonstrated and confirmed that patients with idiopathic thrombocytopenic purpura (ITP), despite low platelet count had elevated levels of platelet derived MPs protecting them from bleeding (106). Increased levels of MPs have been observed in conditions such as diabetes, hypertension, stroke, acute coronary syndromes and hypertriglyceridemia (107). Therefore, MPs may be considered markers of cardiovascular risk (102, 108). Elevated levels have also been found in disseminated intravascular coagulation (DIC)/meningococcal sepsis and are associated with enhanced coagulation (100).

CLINICAL IMPLICATIONS

As mentioned above, sepsis and SIRS, the result of inability in the host infection control, are common and sometimes fatal conditions (37). Also, the I/R injury has an essential role in a number of severe clinical conditions (63, 64).

To study immunomodulation in a human experimental setup as well as in a patient model may provide an increased understanding in the complex mechanisms of inflammation.

AIMS

The overall aim of this thesis was to investigate the potential role of inhaled nitric oxide (iNO) as a modulator of inflammation in humans.

More specifically, the studies aimed to:

- Study the potential anti-inflammatory effect of two different concentrations (30 and 80 ppm) iNO in combination with hydrocortisone (2 mg/kg) in a human endotoxin model as assessed by;
    1. Clinical parameters
    2. Cytokines in plasma
    3. Microparticles in plasma

- Study if the surgical technique performed by tourniquet ischemia in knee surgery with regional anaesthesia is a relevant model for activation of ischemia/reperfusion inflammatory response.

- Study if the inflammatory response, provoked by surgical tourniquet ischemia, could be modulated by iNO 80 ppm.

SUBJECTS AND METHODS

	Individuals	Model	Intervention	Outcome	N=
Study I	Volunteers	Endotoxin	iNO/placebo (N_2) and gluco-corticoids	Symptoms, cytokines in plasma	15
Study II & III	Volunteers	Endotoxin	iNO/placebo (N_2) and gluco-corticoids	Symptoms, cytokines and microparticles in plasma	15
Study IV	Patients	Ischemia/reperfusion	iNO/placebo (N_2)	Adhesion molecules in plasma and muscle	45

ETHICAL CONSIDERATIONS

The protocols for study I-IV were approved by the Human Research Ethics Committee at Karolinska Institutet, Stockholm. The protocols were also approved by the Swedish Medical Product Agency.

The site of the studies was at the department of anaesthesia and intensive care medicine, Karolinska University Hospital, Huddinge, Stockholm.

The volunteers were, before admittance to the study, asked their health state and had a physical and laboratory examination. Written informed consent was obtained from the volunteers as well as from the patients before each study.

EXPERIMENTAL PROTOCOL STUDY I, II AND III

A total of 30 healthy volunteers were investigated in 60 experiments, study III is based on samples taken separately in conjunction with the implementation of study II. Fifteen

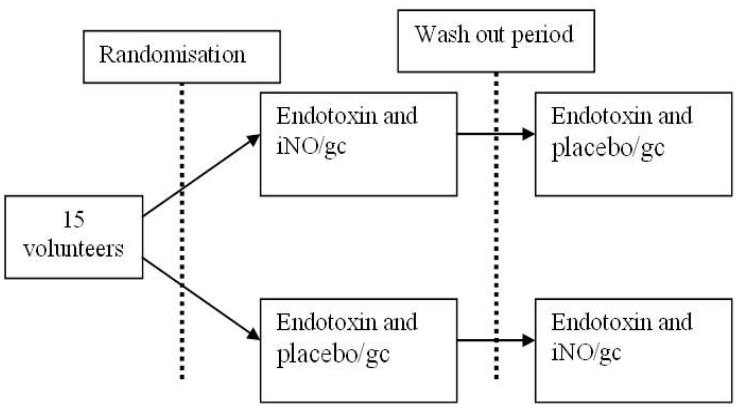

Figure 4. Flow diagram of the design; human volunteers in a cross-over design receiving endotoxin and glucocorticoids (gc) both times, iNO (inhaled nitric oxide) or placebo (inhaled N2) was randomised. Wash out period of 4 and 6 weeks or more in study I and II respectively.

different volunteers were investigated twice in each of the studies I and II. Age range was 19-32 years and they all had normal BMI (range, 18-29), there were 21 men and 9 women.

Intravenous administration of endotoxin and hydrocortisone was given in all of these experiments, while the administration of inhaled nitric oxide (iNO) or placebo (nitrogen, N_2) was randomised and given in a double-blind crossover fashion (Fig. 4). At least 4 weeks elapsed between the experiments as a wash out period. The volunteers all arrived in the morning after having had a light breakfast, they were thereafter put into bed and randomisation took place. Two intravenous lines were inserted, 18 gauge in the cubital fossa for blood sampling, and 20 gauge for infusions (BD Venflon™) in the opposite arm. After a 30 min rest, baseline blood samples were harvested. Tympanic temperature (ThermoScan pro 1, Thermoscan Inc. San Diego, USA), respiratory rate, peripheral oxygen saturation (SpO_2), pulse, ECG monitoring, non-invasive blood pressure (Datex Engström Light®, Helsinki, Finland) were registered. Temperature, respiratory rate, and non-invasive blood pressure were measured every 30 minutes. SpO_2, heart rate and ECG were measured continuously. Nitrogen dioxide (NO_2) was constantly measured (INOvent®) in inspired air as recommended by the manufacturer. Visual Analog Scale (VAS) was used for assessment of nausea, headache and low back pain every 30 minutes throughout the experiment.

SPECIFIC PROTOCOL STUDY I

After baseline samples (at time –0.5 h), endotoxin was infused during 5 minutes (Fig. 5). Infusion with Ringers Acetate started in parallel with the endotoxin injection.

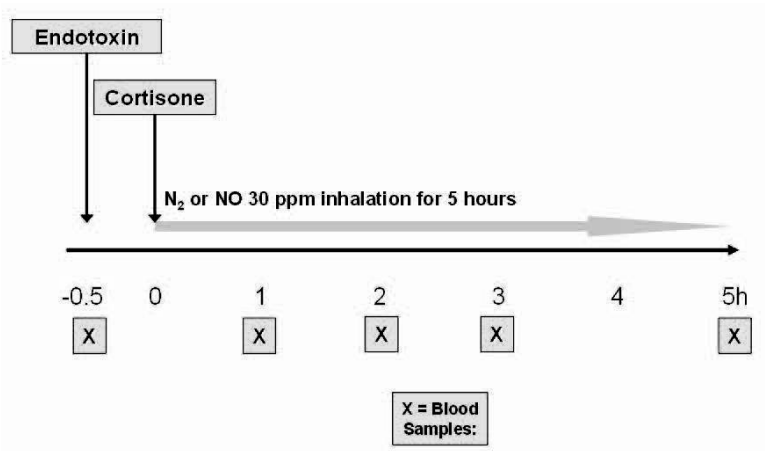

Figure 5. Flow chart of the design in study I.

Hydrocortisone was injected 30 minutes after the endotoxin infusion. Thereafter, at time 0 h, treatment with NO (30 ppm) inhalation (iNO) or placebo gas lasting for 5 hours was initiated. The gas was mixed and administered through a nasal cannula

(SALTER LABS, California, USA). Blood samples were collected at -0.5 hours and at 1, 2, 3, 5 hours.

SPECIFIC PROTOCOL STUDY II AND III

After baseline samples (–2.5 h), treatment with iNO (80 ppm) or placebo gas lasting for 7.5 hours was initiated (Fig. 6).

The gas was mixed and administered through a nasal cannula (SALTER LABS,

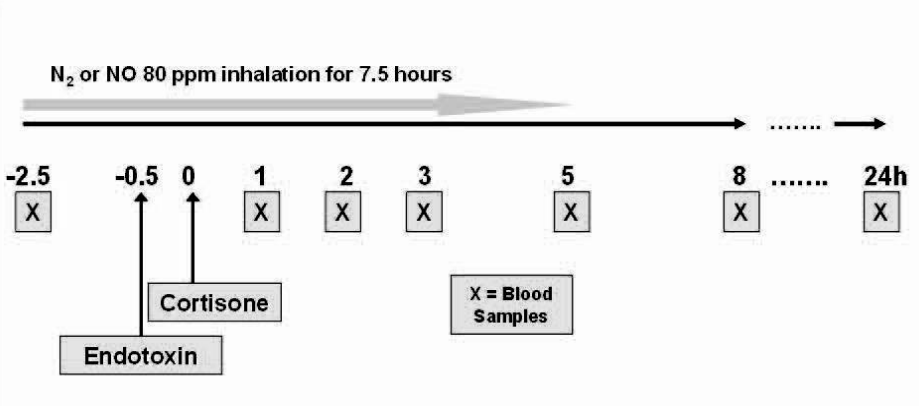

Figure 6. Flow chart of the design in study II.

California, USA). Two hours after the initiation iNO or placebo inhalation, endotoxin was infused during 5 minutes (at time -0.5 h). Infusion with Ringer's Acetate started in parallel with the endotoxin infusion. Hydrocortisone was injected intravenously 30 minutes after the endotoxin injection (at time 0 h). Blood samples were collected at -2.5 hours and at -0.5, 1, 2, 3, 5, 8 as well as 24 hours (every hour in order to measure methaemoglobin).

REAGENTS AND TREATMENTS STUDY I, II AND III

Endotoxin (2 ng/kg BW, Lot nr G3E069, United States Pharmacopeia, Rockville, USA) was mixed with sterile water and treated for 10 minutes by ultrasound (Bransonic 3510, Bransonic Ultrasonic Corp, Danbury, USA) before administration in order to avoid flocculation. Endotoxin was infused during 5 minutes followed by a flush of 20 ml saline.

Infusion with Ringer's Acetate (Baxter Viaflo, Kista, Sweden) 2 ml/kg BW per hour and hydrocortisone sodium succinate (2 mg/kg BW, Solu-Cortef ®, Pfizer, Sweden) was used. NO (final blended at a concentration of 30 and 80 ppm NO respectively in inspired gas, INOmax®, INO Therapeutics, 1060 Allendale Dr. Port Allen, LA 70767, USA) in an oxygen flow of 3 L per minute or placebo (nitrogen, N_2, INO Therapeutics) was administered through the INOvent® delivery system, Datex Ohmeda , Inc. Madison, USA.

BLOOD SAMPLING STUDY I AND II

Blood samples were collected (without a tourniquet) into test tubes containing EDTA. Plasma was collected after centrifugation at 4,000 rpm for 10 min in +4°C and then frozen at –80°C until subsequent analysis. Hematological parameters (white blood cell count, platelet count and haemoglobin) were analyzed within 5 hours at the accredited hospital laboratory. Venous blood samples for methaemoglobin, were measured with ABL800 Flex blood gas instrument (Radiometer Medical, Brønshøj, Denmark).

In study I plasma levels of cytokines IL-1beta, IL-1ra, IL-6, IL-10, TNFα, (Biosource™, Belgium) and RAGE (R&D systems®, Abingdon, UK) were measured using commercially available ELISA kits according to manufacturer's instructions.

In study II plasma levels of cytokines IFN – γ, IL-1beta, IL- 2, 4 5, 8, 10, 12, 13 and TNFα, were measured with an electrochemiluminescence technique (MSD®, Gaithersburg, Maryland, USA). This method allows a small sample volume (25 µ-liters per well) to be used in a multiplex format for several cytokines. ELISA assay kits were used according to the manufacturer's instructions for analysing IL-6 and sRAGE (R&D systems®, Abingdon, UK) and HMGB1 (Shino-Test Corporation, Japan). The latter three were measured at -2,5 hours and at -0,5, 1, 3 and 24 hours.

BLOOD SAMPLING STUDY III

Blood samples were collected (without a tourniquet, in conjunction with study II) at -2.5 hours and at -0.5, 2, 5, as well as 24 hours into test tubes containing 1/10 0.129 M sodium citrate (Fig. 6). Plasma was collected after centrifugation at 4,000 rpm for 10 minutes in +4°C and then frozen at –80°C until subsequent analysis using flow cytometry. The protocol regarding analysis of microparticles is thoroughly described in paper IV. Basically, flow cytometry works using a laserbeam illuminate particles passing in a stream. Since the laser light is scattered by the particles, this scattered light can be used to count and measure the size of the particles. Epitopes can be identified by using an antibody linked to a fluorescent dye. The laser light excites the dye that emits a colour of light that is detected. A computer collects the information from both the scattered and the fluorescence lights (109).

EXPERIMENTAL PROTOCOL STUDY IV

Patients (n=45) planned for knee arthroplasty by one specific orthopaedic surgeon were consecutively included, spinal anaesthesia was used in all cases. The included patients had an age range of 34 - 84 years and a BMI range of 19.6 – 43.0. The patients were randomised into three groups (n=15), and blinded to the patient, surgeon, anaesthetic and laboratory staff. Groups 1 and 3 receiving iNO 80 ppm or placebo throughout the entire operation, whereas group 2 received iNO 80 ppm in the beginning and in the end of the operation, thus with a break in iNO administration during the period of activated tourniquet (Fig. 7).

An intravenous line, 18 or 20 gauge were inserted in the cubital fossa or in the forearm for blood sampling (BD Venflon™). Spinal anaesthesia was performed by an

anaesthesiologist not participating in the study. Thereafter, an oxygen mask, covering mouth and nose, with 5 liter/min oxygen (EcoOxygen Mask, Intersurgical Ltd, UK) mixed with NO or placebo was applied. Blood samples for methaemoglobin were taken

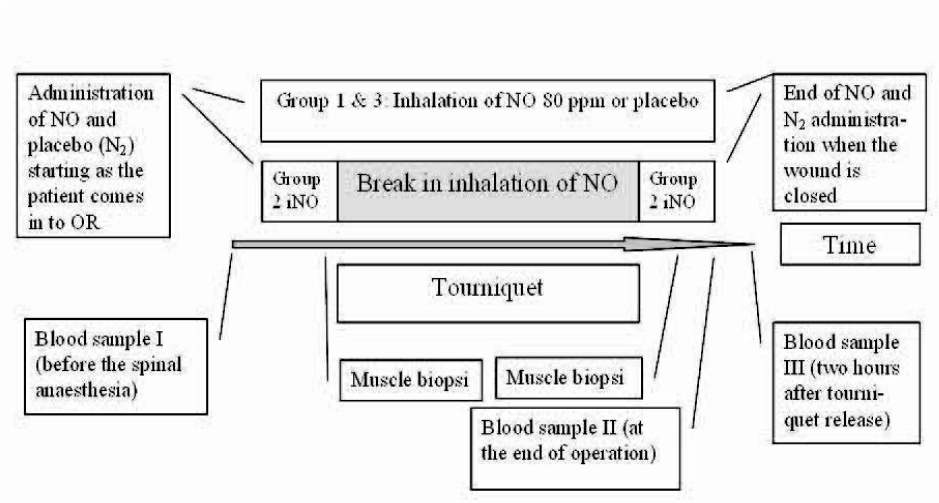

Figure 7. Flow chart of the study design. iNO = inhaled nitric oxide, N_2 = nitrogen (placebo). Group 2 has a break in NO administration during the period of activated tourniquet, administration of NO restarts approximately 2 minutes before the tourniquet release.

hourly. Surgery, and therefore muscle biopsies were performed by the same orthopaedic surgeon in all patients. During the surgery the patients were modestly sedated with propofol (0.5-4.0 mg/kg BW/hour).

TREATMENT STUDY IV

NO (final blended at a concentration of 80 ppm NO in inspired gas, INOmax®, Ikaria®, Inc., Hampton, NJ, USA) or placebo (nitrogen, N_2, Ikaria®, Inc.) was administered through the INOvent® delivery system (Datex-Ohmeda, Inc. Madison, USA).

BLOOD SAMPLING AND MUSCLE BIOPSIES STUDY IV

Blood samples were collected into standard EDTA tubes. Plasma was collected after centrifugation at 4,000 rpm for 10 min in +4°C and then frozen at –80°C until subsequent analysis. Plasma levels of soluble ICAM-1, E-Selectin, P-Selectin and VCAM-1 were measured according to the manufacturer's instructions using a multiplex ELISA kit, (R&D systems®, Abingdon, UK) designed for use with a Luminex® analyser. HMGB1 was analysed using ELISA (Shino-Test Corporation, Japan). Methaemoglobin, as a marker of exposed iNO, was taken and measured hourly with an ABL800 Flex blood gas instrument (Radiometer Medical, Brønshøj, Denmark). Muscle biopsies were immediately frozen in isopentane (C_5H_{12}) with liquid nitrogen (N) and stored at -80°C for subsequent analysis. Biopsies from seven patients in each

group were analysed using immunohistochemistry technique. These were selected from the patients with the best preserved histopathology of the pre-and post ischemic biopsy. The specimens were analysed regarding ICAM (intercellular adhesion molecule), VCAM (vascular adhesion molecule), P-selectin and CD68/macrophages.
The protocols regarding the immunohistochemistry staining process are elaborately described in paper III.

STATISTICAL ANALYSIS

The results are presented as means and standard error of mean the (SEM) for study I-IV if not stated otherwise, ordinal data (VAS) in study I-II are presented as medians and interquartile range. In study I-IV longitudinal data was calculated using two-factor repeated-measures analysis of variance (ANOVA) with post hoc comparisons (Scheffe´) when applicable, using Statistica 5.5 for Windows. For cytokines in study II the area under the curve (AUC) was calculated, and thereafter, a mixed linear effects model was performed, which included sequence (if they had NO or placebo at the first or at the second occasion), to evaluate if tolerance effect existed. For some cytokines, the natural logarithm of AUC was calculated due to outliers and insufficient normal distribution. For the calculations in study II the software SPSS 15.0 was used. Because the VAS data are ordinal in study I and II, and the immunohistochemical data were nominal in study IV, the sign test was used. A P-value of 0.05 was considered to be indicating a significant difference.

RESULTS AND DISCUSSION

This section will present the results as an overview and in brief put them in perspective. Detailed results are given in the separate papers.

EFFECTS OF ENDOTOXIN AND TREATMENT (STUDY I-III)

Clinical condition

All participants felt varying degrees of the typical flu-like symptoms appearing within 60-90 minutes lasting 3 to 4 hours after the endotoxin. No clinical difference between placebo/glucocorticoids (placebo/gc) and iNO/ glucocorticoids (iNO/gc) treatments was found.

Body temperature, heart rate

Body temperature and heart rate increased significantly following endotoxin challenge compared with baseline. However, no differences were seen between the two treatments (placebo/gc and iNO/gc) in either of the two studies (Fig. 8).

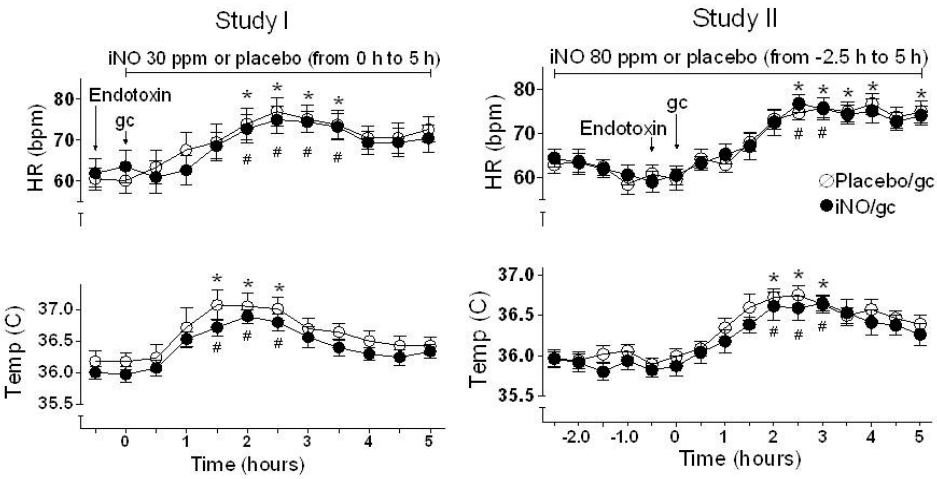

Figure 8. In study I (left) glucocorticoids (gc) 2 mg/kg were administrated and inhaled NO (30 ppm, ●-●) or placebo (nitrogen, ○-○) initiated 30 minutes after endotoxin challenge (2 ng/kg). In study II (right) iNO (80 ppm, ●-●) or placebo (nitrogen, ○-○) was started two hours previous to endotoxin challenge (2 ng/kg) followed by gc 2 mg/kg 30 minutes later. The arrows indicate when endotoxin and treatment were given. HR (Heart rate), Temp (temperature). Mean ± SEM. # * P<0.05 compared with baseline for iNO/gc and placebo/gc respectively.

Haematological parameters

In study II a significant increase in methaemoglobin levels compared with baseline for the iNO/gc treated was observed, as well as a significant difference over time in contrast to placebo/gc treated (Fig. 9). Since methaemoglobin is a redox-product of NO it may be used as marker of exogenously administered iNO (83).

In both studies white blood cell count (WBC) increased significantly over time

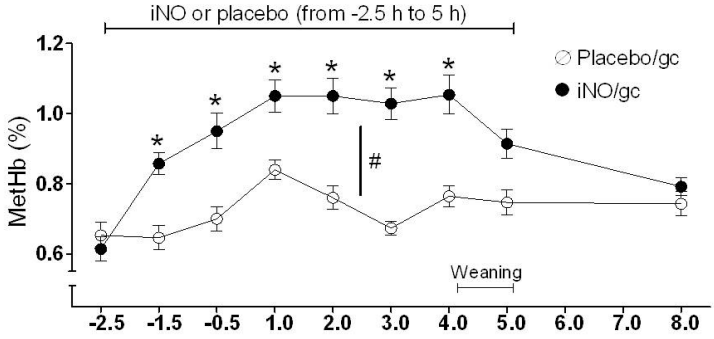

Figure 9. Study II. Significant increase of methaemoglobin levels compared to baseline with the iNO/gc treatment (80 ppm ●-●, * P<0.05) and a difference compared with placebo/gc treatment (nitrogen ○-○, # P<0.05). Mean ± SEM.

	Treatment	-0.5 h	1 h	2 h	3 h	5 h
WBC	iNO/gc	5.5±0.4	4.4±0.4	10.8±1.0*	12.7±0.9*	14.0±0.7*
	placebo/gc	5.5±0.3	5.1±0.9	10.0±0.7*	12.3±0.6*	13.9±0.8*
PC	iNO/gc	238±12	235±15	234±11	235±13	220±12*
	placebo/gc	242±12	231±13	232±11	227±13*	222±11*
Hb	iNO/gc	135±3	136±3	137±3	136±3	135±3
	placebo/gc	138±3	139±3	138±3	138±2	137±2

Table 1. Study I. iNO (inhaled nitric oxide), gc (glucocorticoid), WBC (white blood cell count x 10^9/L), PC (platelet count x 10^9/L), Hb (haemoglobin g/L). Mean ± SEM.
* P<0.05 when compared to baseline values.

compared to baseline, platelet count (PC) decreased significantly in study I but not in study II, no difference regarding WBC or PC was observed between the two treatments (placebo/gc and iNO/gc) in either of the two studies (Table 1 and 2).

	Treatment	-2.5 h	-0.5 h	1 h	2 h	3 h	5 h	8 h	24 h
WBC	iNO/gc	5.3±0.3	5.8±0.4	3.7±0.4	10.2±0.7*	12.3±0.8*	13.7±0.8*	13.1±0.9	7.1±0.5
	placebo/gc	5.4±0.4	5.6±0.5	4.0±0.4	9.7±1.0*	12.1±1.0*	13.9±0.9*	13.9±0.9*	7.1±0.5
PC	iNO/gc	230±11	234±11	217±13	227±13	224±12	217±12	226±11	232±11
	placebo/gc	224±10	224±9	212±10	218±11	216±10	210±12	226±11	232±10
Hb	iNO/gc	139±3	140±3	142±3	141±3	139±3	140±3	141±3	139±3
	placebo/gc	139±3	140±3	142±3	141±3	140±3	141±3	141±3	140±3

Table 2. Study II. iNO (inhaled nitric oxide), gc (glucocorticoid), WBC (white blood cell count x 10^9/L), PC (platelet count x 10^9/L), Hb (haemoglobin g/L). Mean ± SEM. * P<0.05 when compared to baseline values.

Cytokines

In study I endotoxin challenge markedly elevated the plasma levels of TNF-α, IL-6, IL-10, and IL-1ra concentrations versus baseline, but there was no difference between placebo/gc and iNO/gc treatment. sRAGE decreased significantly over time compared to baseline with placebo/gc treatment, whereas during iNO/gc treatment the levels were preserved (Fig. 10).

In study II endotoxin administration elevated the plasma levels of all the measured

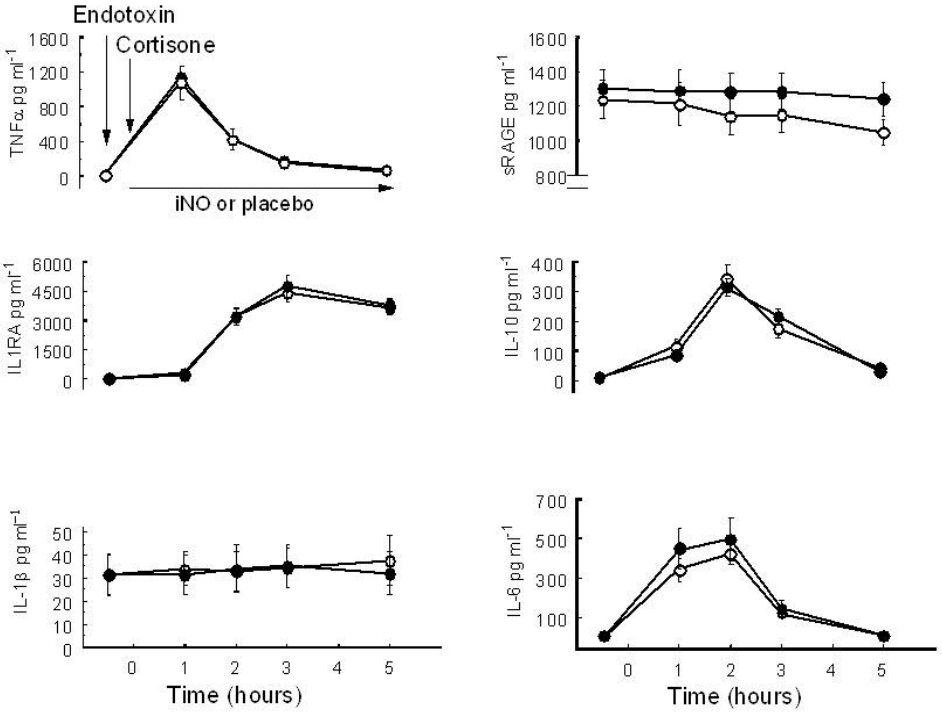

Figure 10. Study I. Cytokine response after endotoxin challenge (2 ng/kg) in healthy volunteers. Treatment with glucocorticoids (single injection, 2 mg/kg) and inhaled nitric oxide (30 ppm, ●-●) or placebo (nitrogen, ○-○) was started 30 minutes after the endotoxin, lasting during 5 hrs. Mean ± SEM.

cytokines with the exception of sRAGE, HMGB1 (Fig. 11) and IL-4 (not shown). As in study I no difference was observed between placebo/gc and iNO/gc treatment.
Endotoxin elicited the expected clinical signs and activation of cytokine levels compared with previous endotoxin administration data in humans not treated with gc (40, 43), however, no significant difference was observed between the iNO/gc and the placebo/gc treated in any of the two studies. Nevertheless, it is not appropriate to compare the values of the cytokines in study I and II with each other. This is due to different types of analytic methods, electrochemiluminescence and ELISA (110). sRAGE was analysed with the same kind of ELISA kit but at a different occasion.

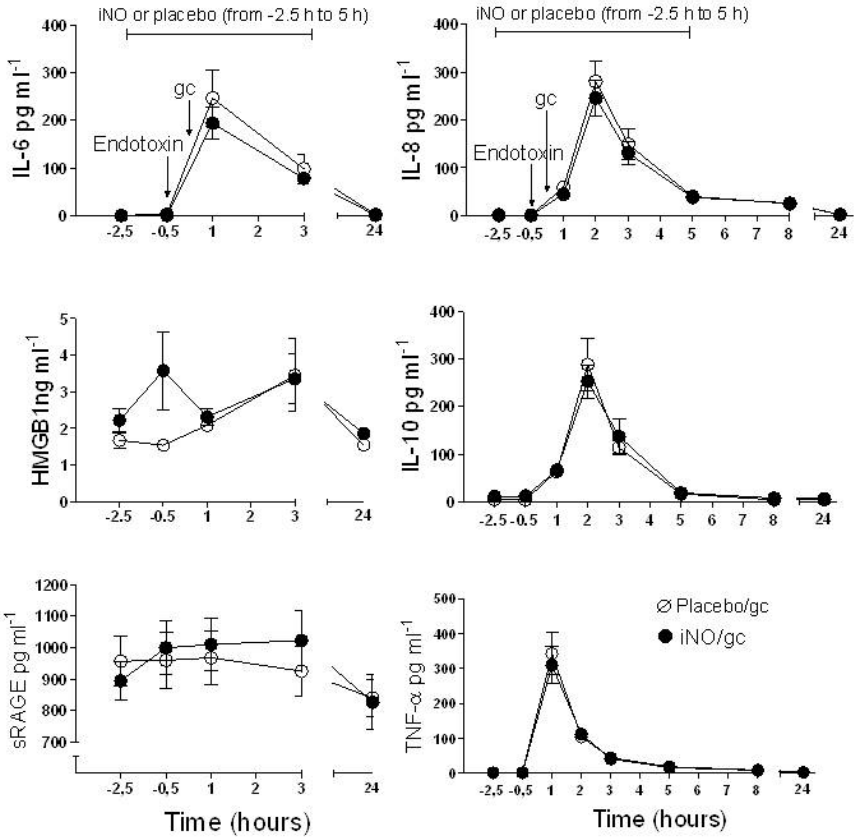

Figure 11. Cytokine response after endotoxin challenge (2 ng/kg) in healthy volunteers. Treatment with inhaled NO (80 p.p.m., ●-●) or placebo (nitrogen,○-○) was started 2 h before endotoxin challenge followed by glucocorticoids (gc) 2 mg/kg 30 min later. Mean ± SEM.

The rational for study I-III is the anti-inflammatory properties of NO in combination with gc as reported by Da et al. Using the combination as a treatment after endotoxin challenge in a porcine model resulted in a blunted inflammatory response in systemic

organs (62). The hypothesis that an interaction between glucocorticoids and iNO could exist may have started from a Chinese study on patients with SARS (severe acute respiratory syndrome). When given a combination of glucocorticoid and iNO an improvement in oxygenation was seen as well as indications on regress in the disease per se, however, the study included only few patients thereby not allowing any conclusions (111). In study II we increased the iNO concentration from 30 to 80 ppm compared to study I, also, we started the inhalation 2 hours before instead of 30 minutes after the endotoxin challenge. Still, no anti-inflammatory effect by the combined therapy, iNO/gc compared to placebo/gc, could be observed in the measured parameters.

The timing of NO administration may be of relevance, since beneficial response was seen when NO was administered early in relation to late in experimental endotoxin ARDS-pig lungs (112) . A dose dependent relation between inhaled NO and systemic effects has been shown in animal (90, 91) and in human models of ischemia/reperfusion injury (93) as well as in an animal model of coronary patency after thrombolysis (113). Also, there is a known difference to equivalent stimulus such as endotoxin in different species (114, 115). Therefore, the discrepancies between doses used in animal models compared to human models are often thousand-fold, for example, pigs may receiving 80μg/kg endotoxin as an infusion for 6 hours (62). Also, animal experiments allow investigation on a cellular level in specific organs e.g. lung, liver and kidney (62).

The method we used in study I-III, repetitive endotoxin challenge in a crossover fashion with the volunteers being their own controls, has been questioned due to tolerance effect. This means that the second endotoxin administration could generate a lower inflammatory response when using a washout period of two weeks (116). However, extending the washout period to 6 weeks has been shown to eliminate this tolerance effect (116, 117).

Microparticles, study III

Phalloidin is a peptide binding to filamentous actin which is only exposed on disrupted cells, thus, phalloidin binding may therefore indicate cell fragments otherwise detected as microparticles (MP) (118). In study III the percentage of particles positive for phalloidin was 10.6 ± 6.9% (mean ± SD), median 10%, indicating acceptable sample handling with low numbers of cell fragments in plasma samples (118) (Fig. 12).

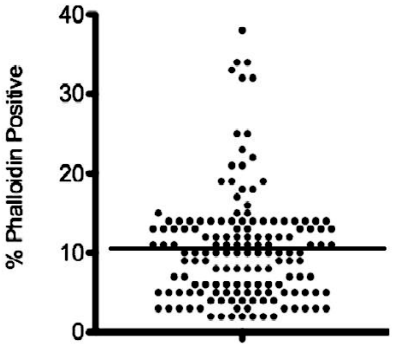

Figure 12. Percental phalloidin positive particles content in the included samples. Horizontal line indicating mean value. Reproduced with permission from Fariborz Mobarrez, Biomedical Scientist, PhD Student

Since MPs have an overrepresentation of phosphatidylserine (PS) on the outer surface compared to ordinary cell membrane, lactadherin that binds to PS is used as a marker for all MP despite cell origin (Fig. 13).

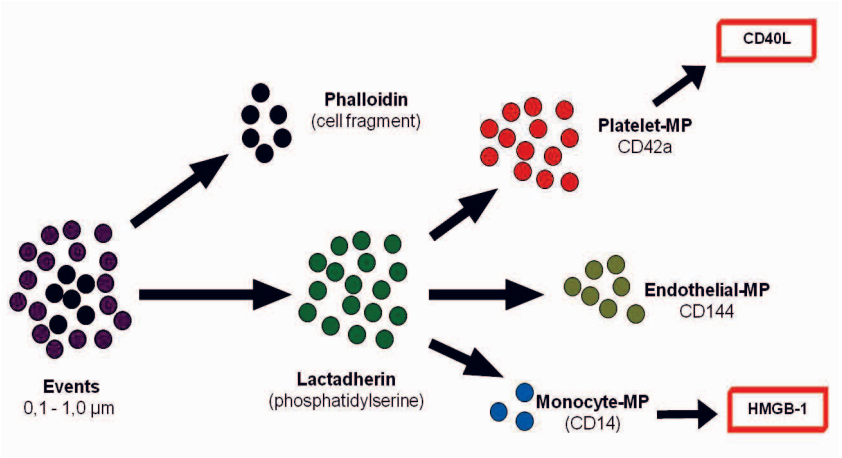

Figure 13. Microparticles incubated with different antibodies. Reproduced with permission from Fariborz Mobarrez, Biomedical Scientist, PhD Student.

In study III all MPs, both total and cell-specific (CD42a - platelet specific and CD14 – monocyte specific), increased significantly compared to baseline at 2.5 and 5.5 hours after endotoxin with the exception of endothelial derived MP (CD144 – endothelial specific) that did not increase over time compared to baseline (Fig. 14). Platelet derived MP (PMP) positive to CD40L increased significantly after 2.5 hours and the same pattern could be seen for HMGB1 positive monocyte MP (MMP) where an early increase was seen after 5.5 hours. No differences were seen between the two treatments (iNO/gc and placebo/gc) regarding impact on MPs levels.

The majority of the MPs in the blood stream emanate from platelets, as observed previously both in healthy humans and septic patients (119, 120).

HMGB1 is secreted extracellulary by monocytes/macrophages in response to proinflammatory stimuli (e.g. LPS, TNFα and IL-1β) (15), but HMGB1 is also passively released from damaged/necrotic (13) and apoptotic cells (17). Hence, the release of HMGB1 and MPs shows important similarities and they both act as important mediators of immune responses (18). We found a significant increase in HMGB1 positive MMPs 5.5 hours after endotoxin challenge, indicating monocyte activation and/or apoptosis. To our knowledge, neither has an increase in HMGB1 been described in a human endotoxin model before (117), nor has it been demonstrated that HMGB1 binds to MMPs. HMGB1 has been described as a late mediator of inflammation in mice (14). The elevation of HMGB1 seen in the current study is in line with that in trauma patients, where a peak was seen 2 - 6 hours after hospital admittance (121). HMGB1 may be bound to PS exposed on MMPs since Rouhiainen et al have shown that HMGB1 bind directly to PS exposed on platelets

(122). We identified only MPs expressing PS, meaning that all MPs measured with our flow cytometry assay have PS molecules on their surface and we found HMGB1 to be elevated in MMPs. However, HMGB1 could also be exposed on MPs other than MMPs since most cells have HMGB-1 present in the nucleus (12). In study II soluble HMGB1 were measured with a commercially available ELISA, although at different time points (1.5 3.5 and 24 hours after endotoxin administration). The lack of alteration of HMGB1 in study II and III may be due to different sensitivities of the assays (ELISA vs flow cytometry), but it is also possible that the peak occurring at 5.5 hours after endotoxin administration, was missed at the 3.5 hour sampling.

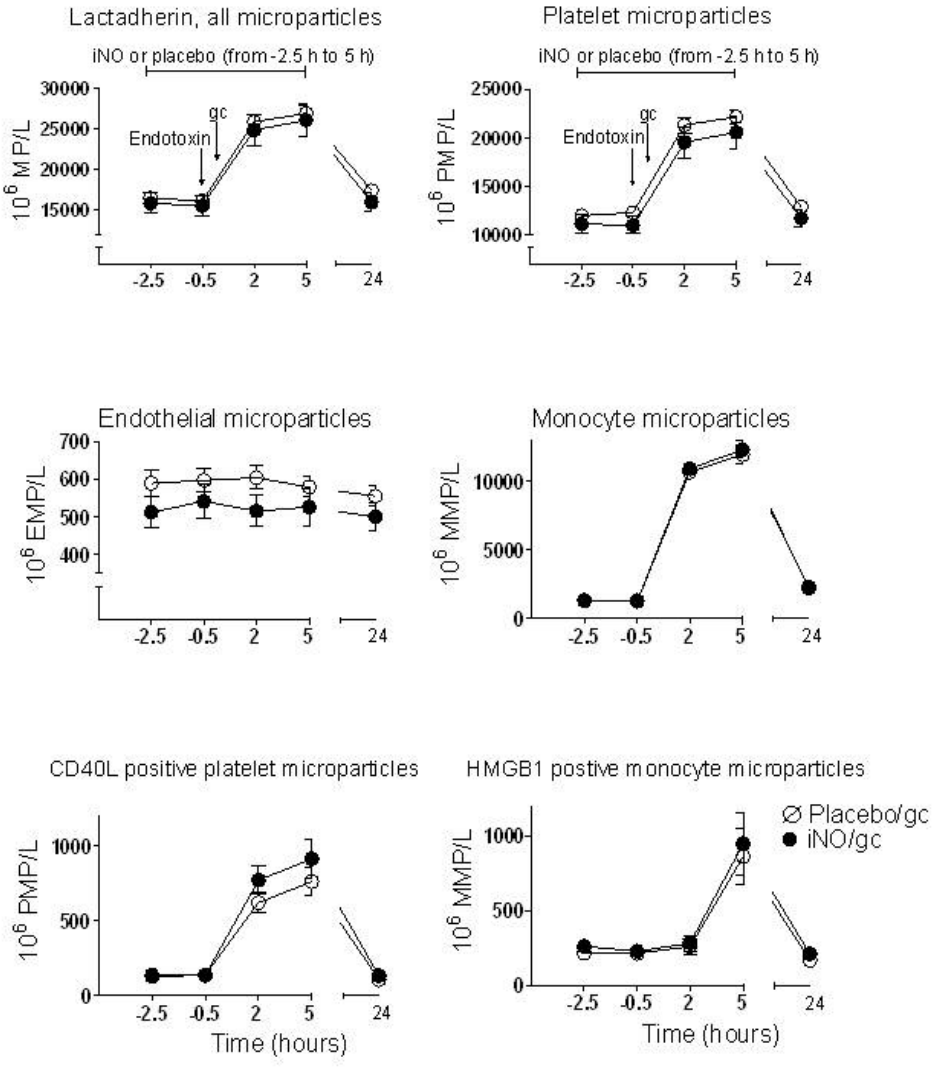

Figure 14. Microparticles after endotoxin challenge in healthy volunteers. Endotoxin was administered at -0.5 hours and glucocorticoids (gc) at 0 hours. Treatment in a double-blind randomized, crossover fashion regarding inhaled NO or inhaled placebo (nitrogen, N_2). Mean ± SEM.

The endotoxin challenge resulted also in CD40L expression on PMPs, to our knowledge an observation not earlier described. This molecule (CD40L, CD40 ligand or CD154), originally found on stimulated T-cells, are also expressed on platelets (123). Interaction between CD40L and the receptor CD40 cause both pro-inflammatory and pro-thrombotic activities including the induction of endothelial cells secreting chemokines and expressing adhesion molecules (123-125). Furthermore, CD40L stimulate expression of tissue factor in endothelial cells (126) and reduction of CD40L on platelets as well as in its soluble form is associated with a reduced thrombin generation (124). Thus, an increase in the number of PMPs and increased PMPs exposing CD40L is likely to reflect platelet activation (124).

The increase in CD14+ monocyte MPs are in consistence with the findings by Aras et al in healthy volunteers receiving endotoxin, where a return to normal levels was observed within 8 hours (127). In contrast to platelet and monocyte MPs, endothelial MPs measured with CD144 (VE-Cadherin) were not found to be elevated. Aras et al found elevation in CD144 positive EMPs in 3 out of 18 healthy volunteers receiving endotoxin, a finding which we interpret as similar to ours, i.e. no significant effect (127). In studies on sepsis patients EMPs have been shown to lead to various results, an observation that may depend on the use of different antibodies (119, 128). The use of CD144 in our study may be a limitation, since this molecule may provide limited sensitivity to detect EMPs. The combination with other antibodies may increase the sensitivity to detect endothelial cell activation (129). Another reason for the disparity between different studies regarding levels of EMPs, may relate to when the samples were taken in relation to the course of the septicemia. The human endotoxin model makes it possible for us to study the whole time-pattern of the inflammatory response compared to septic patients, where the beginning of the course of the disease often already has occurred when the patients are admitted to the hospital.

STUDY IV

Endothelial cell activation and inflammation in this study was evaluated using two standard methods, ELISA and immunohistochemical techniques. Plasma levels of HMGB1, P-selectin, VCAM, and E-selectin all remained unaltered with no differences between groups. However, soluble ICAM showed a small, but significant, decrease in group 1 and 3 (Fig. 15).

In addition, when analysing the muscle biopsies regarding the expression of adhesion molecules (ICAM, VCAM, P-selectin) and CD68/macrophages, no differences were observed between the three groups or within the separate groups before and after ischemia.

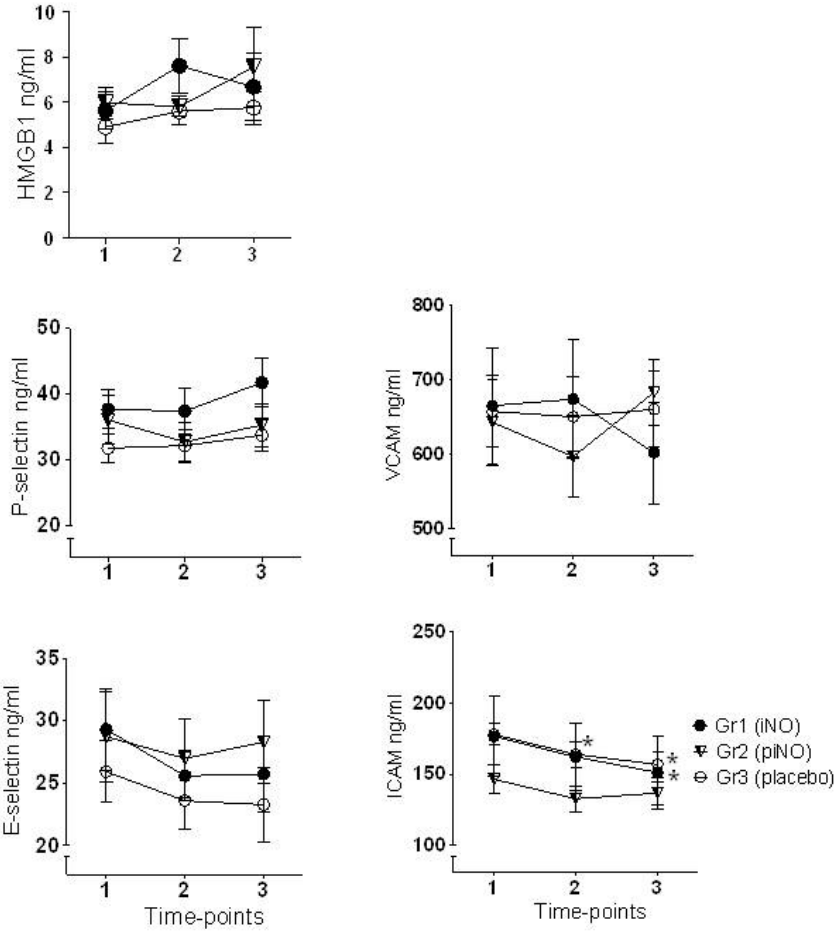

Figure 15. Effects on soluble adhesion molecules and HMGB1 in plasma from patients. A significant decrease in ICAM was observed in group 1 (iNO) at time-point 3 (two hours after tourniquet release) and in group 3 (placebo) at time point 2 (at the end of operation) and 3. No differences were observed between the groups. * P<0.05 compared with baseline. Mean ± SEM.

As in study II methaemoglobin increased significantly over time in the iNO treated groups compared to the placebo treated, thus, serving as a marker of exposure to iNO (83) (Fig. 16).

Surprisingly, no signs of endothelial cell activation or an inflammatory response could be observed neither in plasma nor in muscle biopsies in this clinical human study with reperfusion following an ischemic event. As a consequence, no influence was seen nor could be expected, regarding the randomised iNO exposure on the measured parameters. Our result are in contrast to Mathru et al who found, in anaesthetised patients going through knee surgery, significantly increased inflammatory parameters in blood and muscle that were attenuated by the administration of iNO (93). Although

total tourniquet duration in the present study was 60-140 minutes, which is in concordance with the approximately two hours presented by Mathru et al, some differences between our and Mathru´s study may be pointed out (93). Firstly, in the present study the patients all had spinal anaesthesia with propofol sedation in concordance to the clinical routine, in contrast to Mathru et al who used general anaesthesia with thiopental and isoflurane. The choice of anaesthetic method may have importance, since different anaesthetic drugs have been reported to have influence on inflammation in various settings. For example, isoflurane anaesthesia has been found to impair renal function in an ovine endotoxin study (130), isoflurane has also been demonstrated to protect the hepatic tissue compared to penthobarbital sodium in an rat I/R model (131), whereas isoflurane did not attenuate the liver damage ketamine did in an endotoxin rat model (132). Also, propofol has in the I/R setting been proposed to possess antioxidant capabilities, both in rats (133) and in humans (134-137). However, lack of beneficial effects against myocardial I/R injury (rat) using propofol has also been reported (138). Further, in patients undergoing coronary artery bypass graft surgery having remote ischemic precondition, propofol anaesthesia could not, compared to isoflurane decrease myocardial damage (139). Hence, there are several inconsistencies regarding the effect on inflammation by different anaesthetic methods in the inflammatory setting. Since we used propofol sedation in addition to the spinal anaesthesia it could be speculated that this combination may have influenced the inflammatory response in the ischemic tissue.

Secondly, our patients were almost twice as old compared to patients in the Mathru

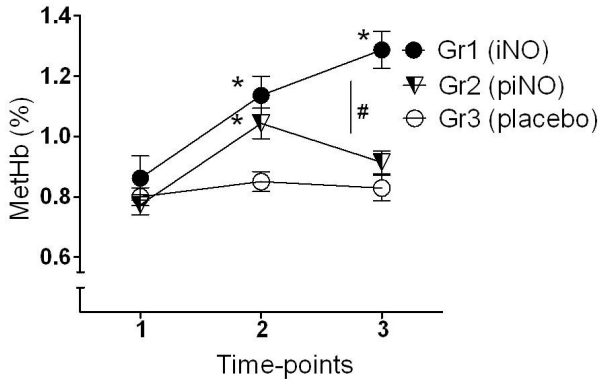

Figure 16. There was a significant increase in methaemoglobin levels compared with baseline for the iNO treated patients as well as a significant difference over time in contrast to the placebo treated (* P<0.05), also, a significant difference between the two iNO groups was observed (# P<0.05). Group 1 (iNO) and 3 (placebo, N_2) receiving iNO 80 ppm or placebo throughout the entire operation, whereas group 2 (piNO) only received iNO 80 ppm in the beginning and in the end of the operation. Time-point 1; before the spinal anesthesia, time-point 2; before tourniquet activation, time-point 3; after tourniquet release. Mean ± SEM.

study (93). A positive correlation between age and the plasma concentration of soluble ICAM and VCAM has been observed in humans (140). Also, in rats the expression of soluble adhesion molecules has been reported to increase during aging, and when treated with LPS a more pronounced expression was seen in older animals (141). Therefore, it seems unlikely that the higher age in the present study could explain the difference in the inflammatory response.

HMGB1 has been shown to be of vital importance in mediating injury in settings of I/R, for example, in mice HMGB1 protein expression in the liver was up-regulated one hour after reperfusion, and after 30 minutes in the heart (21, 23). In patient studies with cerebral and myocardial ischemia respectively, elevated levels of HMGB1 were seen within 24 hours (24, 25). The levels of HMGB1 in the present study are in the expected normal range (117, 142).
A decrease in soluble ICAM was observed over time in the placebo group and in the group receiving iNO throughout surgery, a finding corresponding with an earlier study by Huda et al (143), where a decrease in soluble ICAM was seen post-tourniquet in patients undergoing knee surgery. Notably this was seen in conjunction with an increase in ICAM mRNA in muscle biopsies (143). In our study the ICAM staining in the muscle biopsies does not confirm this decrease in soluble ICAM. Followingly, we do not interpret these small changes as clinically relevant.

In this study we administrated a maximal dose of iNO in order to evaluate the hypothesised anti-inflammatory effect, throughout the whole period of surgery or with a break when the tourniquet was activated. This design was used in order to investigate if iNO administered when there was circulation to the leg could modulate the inflammatory response, hypothesising that the administration of iNO may create a repository of NO (87, 88).

CONCLUSIONS

Based on the experiments performed in this thesis the following conclusions were made:

- In the human model of volunteers receiving 2 ng/kg endotoxin, the clinical symptoms and increased cytokine activation in plasma are in agreement with current knowledge.

- In this human endotoxemia model, the combination of intravenously administered glucocorticoids and inhaled nitric oxide, using a low dose (30 ppm) and a maximal dose (80 ppm), elicited no anti-inflammatory effects with regard to;

  1. Clinical symptoms

  2. Cytokines in plasma

  3. Microparticles in plasma

- The endotoxin infusion in healthy volunteers resulted in an increase in microparticles released from platelets and from monocytes but not from the endothelium.

- The endotoxemia generated an early increase of the activation markers HMGB1 on monocyte microparticles and CD40L on platelet microparticles.

- In patients undergoing knee arthroplasty in spinal anesthesia, the ischemia/reperfusion created by a tourniquet did not cause any signs of endothelial cell activation or inflammatory response measured as levels of adhesion molecules and macrophages in muscle biopsies as well as HMGB1 and soluble adhesion molecules in plasma. Hence, no influence was seen nor could be expected, regarding the randomised iNO exposure on the measured parameters.

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