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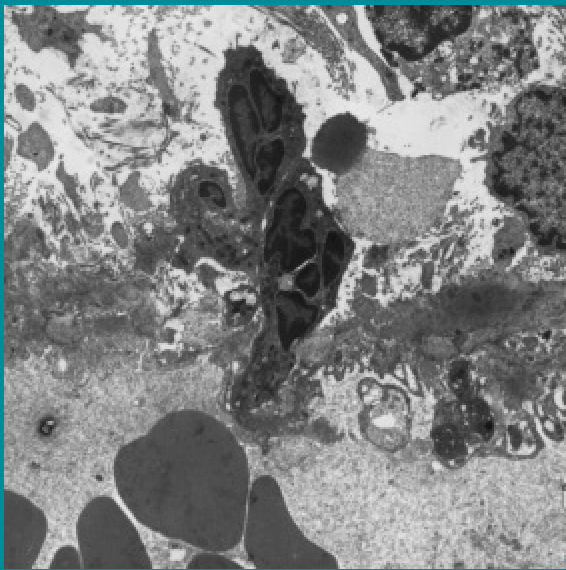
EXPERIMENTAL SKIN FLAPS AND NITRIC OXIDE



Örjan Gribbe

Karolinska Institutet

From
The Department of Physiology and Pharmacology, Karolinska Institutet
and
The Department of Molecular Medicine and Surgery,
Section of Plastic and Reconstructive Surgery, Karolinska Institutet
Stockholm, Sweden

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Örjan Gribbe
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by Örjan Gribbe

Abstract. Surgical flaps are used in plastic surgery to reconstruct tissue defects due to trauma or cancer removal. Occasionally flaps are subjected to ischemia and reperfusion injury leading to flap failure.

Nitric oxide (NO), a small gaseous molecule, has vast physiological importance as it participates in the regulation of blood pressure, blood flow, neurotransmission and immune response. NO is synthesized by the enzyme NO synthase (NOS), which exists in both constitutive and inducible forms. Constitutive NOS in endothelial cells (eNOS) continuously synthesizes NO in small amounts causing vasodilation and inhibition of platelet and leukocyte activity. Inducible NOS (iNOS) in leukocytes and inflamed tissue synthesizes NO in large amounts, which under certain circumstances leads to tissue destruction.

Ischemia and reperfusion injury has great clinical impact and affects tissues such as the brain (stroke), heart (myocardial infarction) and surgical flaps (necrosis). The mechanisms underlying this tissue damage are not fully understood and methods to prevent and treat flap necrosis would be of great clinical value.

In the present thesis experimental flaps in the rat were studied with special reference to the role of NO. Different experimental skin flap models in the rat were used. NOS activity, flap ultrastructure, flap blood flow and flap survival after modulation of NOS and administration of NO were studied.

Constitutive NOS activity was demonstrated in intact skin by citrulline assay. In an ischemic dorsal random flap model this constitutive NOS gradually decreased after flap surgery. Concurrently, increasing signs of endothelial damage and accumulation of leukocytes and platelets was observed by transmission electron microscopy. Inhibition of the constitutive NOS led to a decreased flap blood flow, as measured by laser Doppler technique, and also to a decrease in flap survival.

Intact skin did not display any iNOS activity, whereas in the dorsal flaps iNOS activity was seen to gradually appear after surgery. At the same time an accumulation and extravasation of leukocytes was seen. Treatment with dexamethasone was found to prevent the induction of iNOS and also to increase flap survival.

Besides enzymatic formation of NO, non-enzymatic formation through the reduction of nitrite (NO_2^-) under acidic and reducing conditions has been described. With this knowledge at hand, a cream containing increasing concentrations of NO_2^- and vitamin C was mixed and applied to the surface of an epigastric flap model. The cream generated NO in a concentration dependent manner, as measured by chemiluminescence, and increased the supplying and superficial blood flow in the flaps, as measured by transit-time ultrasound technique and laser Doppler technique respectively. Furthermore, the gas nitrogen dioxide was generated by the cream.

Taken together, the results show that constitutive NO, probably mainly derived from eNOS, is important for flap survival as it maintains blood flow and possibly also inhibits accumulation, aggregation and activation of leukocytes and platelets. Furthermore, the results indicate that induction of iNOS, which is capable of producing large concentrations of NO, could be negative for flap tissue. NO at high concentrations has previously been demonstrated to be tissue destructive, both in itself and also through the formation of different free radicals. Inhibition of the negative effects of NO and administration of NO to counteract a decrease in endogenous, constitutive NO synthesis could prove beneficial to flap tissue and might become useful in a clinical setting. Local administration, for example through the application of a cream to the flap surface, is an interesting and attractive way of treatment.

Keywords: Surgical flaps, nitric oxide, ischemia, reperfusion, nitrite, laser Doppler, transit-time ultrasound flowmetry, electron microscopy, NO donors, NOS inhibitors
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A graft is a piece of detached skin which is dead when you put it on and comes to life later.
A flap is a partly attached piece of skin which is alive when you put it on and may die later.

Sir Harold Gillies, circa 1920

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Gribbe Ö, Lundeberg T, Samuelson U E and Wiklund N P
Nitric oxide synthase activity and endothelial ultrastructure in ischaemic skin-flaps
(British Journal of Plastic Surgery. 1997;50(7):483-90)

II. Gribbe Ö, Lundeberg T, Samuelson U E and Wiklund N P
Dexamethasone increases survival and attenuates induction of inducible nitric oxide synthase in experimental skin flaps
(Annals of Plastic Surgery. 1999;42(2):180-4)

III. Gribbe Ö, Samuelson U E and Wiklund N P
Effects of nitric oxide synthase inhibition on blood flow and survival in experimental skin flaps
(Journal of Plastic, Reconstructive and Aesthetic Surgery. In press)

IV. Gribbe Ö, Gustafsson L E and Wiklund N P
Transdermally administered nitric oxide by application of acidified nitrite increases blood flow in rat island flaps
(Submitted to European Journal of Pharmacology)

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To my family

CONTENTS

ABBREVIATIONS

BH_2	dihydrobiopterin
BH_4	tetrahydrobiopterin
cGMP	cyclic guanosine monophosphate
D-NAME	D^{ω}-nitro-L-arginine methyl ester
EDRF	endothelium-derived relaxing factor
eNOS	endothelial nitric oxide synthase
GTP	guanosine triphosphate
H_2O_2	hydrogen peroxide
Hb	hemoglobin
iNOS	inducible nitric oxide synthase
L-Arg	L-Arginine
L-NAME	N^{ω}-nitro-L-arginine methyl ester
LTB_4	leukotriene B_4
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO_2	nitrogen dioxide
NO_2^-	nitrite
NO_3^-	nitrate
NOS	nitric oxide synthase
NO_x	NO plus NO_2 (and other nitrogen oxides)
O_2^-	superoxide
OH	hydroxyl radical
$ONOO^-$	peroxynitrite
sGC	soluble guanylyl cyclase
SOD	superoxide dismutase
TEM	transmission electron microscope / microscopy
TXA_2	thromboxane A_2

INTRODUCTION AND GENERAL BACKGROUND

1. History and development of flap surgery

Random and axial flaps

The history of flap surgery dates back as far as 3000 years. Sanskrit texts from India tell of pedicled pieces of skin from the chin and forehead used for the reconstruction of defects on the nose [1].

A flap can be defined as a piece of autologous tissue such as skin, fat, muscle, bone, intestine or omentum, which carries its own blood supply and is moved to cover a local or distant defect caused by for example trauma or tumour surgery.

Blood supply is essential for the survival of flap-tissue. The skin flaps used in ancient India were so-called random flaps, which in fact was the only common flap-type until the 20^{th} century. Random means that they do not receive their blood supply by defined vessels but instead rely on a network of small blood vessels in the dermal and some times also subdermal plexuses [2, 3]. Random skin flaps have to be created carefully with regard to their length-width ratio in order for the distal part of the flap, the tip, to survive. In 1893 Dunham and in 1898 Monks as well as Brown described skin flaps that included the temporal artery, for the reconstruction of chin, nose and ear defects respectively [4-6]. This new type of flap, which unlike the random skin flap has a defined blood supply, did however not become popular until the second half of the 20^{th} century. In 1962 Bakamjian, by coincidence, discovered a skin flap for pharyngoesophageal reconstruction, based on perforators from the internal mammary artery [7] and soon thereafter, Milton emphasised the value of a flap having a defined blood supply [8]. Extensive research on flaps with a defined blood supply was performed by Daniel [9] and subsequently by McGregor, who came up with the name "axial flaps" [10].

Muscle and musculocutaneous flaps

The history of muscle flaps probably does not go as far back as that of skin flaps, but in 1896 Tansini described the latissimus dorsi muscle flap for breast reconstruction [11]. Again the technique fell into oblivion, and except for sporadic reports [12, 13], muscle flaps did not become popular until the 1970s through the works of Ger and Vasconez in the reconstruction of defects on the lower leg [14, 15].

Towards the mid 20^{th} century, many plastic surgeons had realised the importance of the musculature as a carrier of blood supply to the overlying skin [16-20] However, it was only in the late 1970s that McCraw rediscovered and extensively described the musculo-cutaneous flaps and the "independent myocutaneous vascular territories" both in animals and man [21, 22]. The vascular anatomy of muscles and a classification of musculocutaneous flap were later presented by Mathes [23].

Fasciocutaneous and perforator flaps

The skin's blood supply was described by Spalteholz in 1893 as consisting of direct (dominant) or indirect (supplementary) vessels [24, 25]. The direct vessels are often large, either passing between or through the muscles (musculocutaneous vessels), on their way to the skin. The indirect vessels first supply other structures such as muscles and then terminate in the skin as small branches. After Milton's rediscovery of the importance of a defined blood flow for flap design [8], the interest in the blood supply of the skin rose. In 1981, Pontén designed a skin flap with a defined blood supply but without muscle. He realised the importance of the circulation in the muscle fascia and created a flap on the lower leg containing vessels, muscle fascia, subcutis and skin [26]. This was the first example of a fasciocutaneous flap and with this knowledge it became possible to safely raise large flaps without the need to include the underlying muscle. His co-workers named the flaps the "Pontén super flaps" [27, 28]. Cormack later classified the fasciocutaneous flaps [29] as did Nakajima [30]. Nakajima described the blood supply to the skin and categorized the fasciocutaneous flaps into six different subgroups according to the way the supplying artery, the "musculocutaneous perforator" enters the skin [30]. In addition, Taylor extensively described the circulation to the skin, mapping the different areas of the body "angiosomes" and how each area is supplied by specific arteries [31].

The next revelation in flap-surgery was the discovery that not even the muscle fascia is always needed for the skin's blood supply. The skin and subcutis can instead rely on the direct blood supply from an artery perforating the muscle fascia. Thanks to this finding, the perforator flap was born and has subsequently been widely recognised. Beak and later Song described the medial and lateral thigh flaps consisting of only skin and subcutis [32, 33] and the anterolateral thigh flap is now extensively used in the clinical setting [34]. Koshima was first to describe the deep inferior epigastric flap (skin and subcutis from the belly) and used this flap in reconstructions in the head and neck [35]. Allen later modified and extended this flap using it for breast reconstruction [36].

Some confusion persists in the classification of perforator flaps and some argue that only flaps receiving their blood flow from vessels that perforate muscles should be included [37]. Consensus has however been reached at different international meetings [30, 38, 39].

Free flaps

The flaps described above can all be used as local, pedicled flaps to cover defects in proximity to the site of flap harvest. However, when there is no suitable flap in the area close to the defect, other solutions have to be found. During the two world wars, a technique using tube-shaped pedicled flaps was adopted. In this procedure, for instance, a tubular flap from one leg would be connected with a defect on the other leg (cross-leg flap) or a tubular flap from an arm would be connected to a defect in the face. The patient would then have to keep the body parts still (often fixated by a cast) for days or weeks, until blood vessels from the recipient area had grown into the flap and the pedicle could be cut. This situation changed dramatically with the invention of microvascular surgery.

Microvascular surgery evolved through the merging of vascular surgery and microsurgery. In 1877, Eck performed the first vascular anastomosis when he created a porta-caval shunt in the dog [40] and the technique was later used by Pavlov (yes, the man with the conditioned reflex dogs) [41]. Just before the turn of the 19^{th} century, Murphy described the first vascular anastomosis in man [42] and in 1902 Carrel presented the "triangulation method" for end-to-end anastomosis making it possible to suture small vessels [43]. Thanks to the invention of the microscope in the late 19^{th} and beginning of the 20^{th} century [44], the ear, nose and throat (ENT) surgeons Nylén and later Holmgren were able to perform the first microsurgical operations in the first years of the 1920s [45].

Modern microvascular surgery was born when Jacobsen and Suarez borrowed an operating microscope from ENT colleagues to perform numerous successful anastomoses in small vessels [46]. Krizek soon performed the first free skin flap in an animal [47] and in 1973, Taylor and Daniel performed the first human microvascular transfer of a superficial groin flap and coined the term “free flap” [48]. The technique spread quickly during the 70s and 80s and came to revolutionise plastic surgery. The complicated tubular flaps suddenly became history. Soon microvascular transfer of bone (fibula to tibial defect) [49], muscle (gracilis to face) [50] and small intestine (jejunum to esophagus) were performed [51]. The invention of fasciocutaneous and recently, perforator free flaps, has further developed microvascular surgery making it possible to replace skin with skin, “like with like”.

Combined flaps

As already described, flaps are often combinations of different tissues. Flaps can thus contain skin, subcutaneous tissue (fat), muscle fascia, muscle, periosteum and bone. An example of a musculocutaneous flap is one containing the latissimus dorsi muscle and the overlying skin (and subcutaneous fat). The flap may be used either as a pedicled flap for breast-reconstruction after breast cancer [11, 52], or as a free flap in for example reconstruction of a severe traumatic wound on the lower leg [53]. An example of a free flap containing skin, subcutaneous fat, muscle fascia, muscle, periosteum and bone is the osteomyocutaneous fibular flap, which for example can be used for the reconstruction of the mandible and surrounding soft tissue after cancer removal [54]. Recently microvascular surgery has reached new heights with transplantation of one hand [55], both hands [56] and one year ago, parts of the face [57].

Flap failure

A serious complication in flap surgery is partial or total failure and necrosis of the flap. This causes mild to severe discomfort for the patient and on occasion leads to reoperation. At worst, the outcome is fatal due to severe infections or rupture of exposed vessels [58].

In free flap surgery a success rate of 91-99% has been reported [59-63] with variations depending on the flap type [63]. When free flap failure occurs, it is often complete and caused by thrombosis at a vascular anastomosis during the first three postoperative days in either the supplying artery, the draining vein or in some cases, both vessels [64, 65]. If found in time, the flap can be salvaged through reoperation and resuturing of the anastomoses. When failure occurs in random pedicled flaps it is often only partial, affecting the distal, most ischemic end of the flap. Partial failure is sometimes also seen in axial pedicled flaps and free flaps and when present mostly affects the distal parts of the flaps where the circulation at times is of a random nature.

On some occasions the cause of flap failure is evident. Examples are hematoma, an infection or a tight dressing strangling the flap’s circulation. Planning and surgical skill are important and in free flap surgery, patent anastomoses and short ischemia time during surgery is crucial [66]. Patient factors such as diabetes, cardiovascular disease and, in some studies old age, have been shown be negative to flap survival [67-70]. Also, both experimental [71] and clinical studies have shown that smoking increases flap failure through vasoconstriction and increased frequency of thrombosis [72-74]. However, even when surgery is performed correctly and no complicating factors are present, flap necrosis is sometimes seen.

Experimental flaps in laboratory animals
The mechanisms leading to flap failure are not fully elucidated. However, as will be described later, ischemia and reperfusion play a central role. An increased understanding of the physiology and pathophysiology in flap tissue would be of great value as it could lead to the development of new methods to prevent and treat flap failure. For this purpose both random and free flap models, with controlled and reproducible flap ischemia and necrosis, have been designed in different laboratory animals such as rats [75, 76], pigs [77] and mice [78]. By far the most frequently used laboratory animal in flap research is the rat [75, 76].

2. NO: discovery and physiology

In order to put flap failure and necrosis into a modern perspective the role of nitric oxide and other factors during ischemia and reperfusion will be discussed later. First follows a historical background on nitric oxide and a brief description of its physiology.

Nitroglycerin
In 1846 the French chemist Théophile-Jules Pelouze founded a laboratory school in Paris where experiments on explosive materials such as guncotton and other nitrosulphates were performed. A year later one of his students, the 35-year-old Italian Ascanio Sobrero, discovered the highly explosive substance nitroglycerin. Apart from its explosive qualities, Sobrero also noted that "a very minute quantity put upon the tongue produces a violent headache for several hours" [79]. This is probably the first description of the biological effects of the diatomic gaseous molecule nitric oxide (NO).

The discovery of NO as a biological mediator
130 years after the discovery of nitroglycerin, the American pharmacologist of Albanian descent, Ferid Murad, was focusing his research on the second messenger cyclic guanosine monophosphate (cGMP), which is formed from guanosine triphosphate (GTP) by the enzyme guanylyl cyclase. In studies on different "nitrovasodilatators", as Murad and his co-workers called them, they found that NO stimulated guanylyl cyclase in smooth muscle cells and increased cGMP levels thus causing smooth muscle relaxation. As a result, in 1977 Murad provided the first evidence of a biological effect of NO [80]. A couple of years later, in 1980, Robert Furchgott described the obligatory role of endothelial cells in smooth muscle relaxations induced by acetylcholine. He showed that the endothelium needs to be intact in order for acetylcholine to exert its relaxing effect on smooth muscle in aortic preparations [81]. By removing the endothelium, the relaxing effect of acetylcholine was abolished and he postulated that the endothelium released a substance, which he later named endothelium-derived relaxing factor, EDRF [82]. The quest for the identity of EDRF now started and Murad's group went on to show that EDRF increases the levels of cGMP [83] in smooth muscle cells causing relaxation and suggested that EDRF be an "endogenous nitrite or nitrovasodilatator" [84]. Shortly thereafter in the summer of 1986 at a meeting in Rochester, Minnesota, Furchgott and Louis Ignarro independently reported that EDRF and NO have similar actions [85, 86] and finally, in 1987, Salvador Moncada's group demonstrated direct evidence of the release of NO from endothelial cells through the use of chemiluminescence [87].

The discovery of NO in the endothelium set off an avalanche of research and to date over

35000 articles with the words “nitric oxide” in the title and over 78000 with “nitric oxide” in the title or abstract have been published (www.pubmed.gov).

NO synthesis by NOS

NO is synthesized by different isoforms of the enzyme NO synthase (NOS) through the conversion of the amino acid L-arginine (L-Arg) and molecular oxygen to the amino acid L-citrulline and NO. In addition, NADPH is oxidized to $NADP^+$. The reaction was first described to occur in the endothelium by endothelial NOS, eNOS [88] and the enzyme was subsequently found in neurons (neuronal NOS, nNOS) and macrophages (inducible NOS, iNOS) [89]. nNOS was the first isoform to be purified and cloned [90, 91].

The two isoforms eNOS and nNOS are constitutive, continuously synthesizing NO in small amounts (picomolar range). The third isoform, iNOS requires de novo synthesis after the induction by proinflammatory cytokines (e.g. interleukins, tumour necrosis factor, interferons) or bacterial lipopoly-saccharides (endotoxins). Once activated iNOS produces NO at high concentrations (nanomolar range) [92].

NOS consists of two identical chains (homodimers), each containing two domains: a C-terminal reductase domain and an N-terminal oxygenase domain. The two constitutive forms eNOS and nNOS bind calmodulin loosely and are activated when ionic calcium (Ca^{2+}) binds to calmodulin. The inducible form iNOS however, binds calmodulin tightly and thus does not need Ca^{2+} to be activated. The constitutive forms eNOS and nNOS are accordingly said to be Ca^{2+} dependent whereas the inducible form iNOS is designated Ca^{2+} independent [93].

Besides NADPH and calmodulin, NOS requires the co-factors tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and haem to function properly [93].

Mechanisms behind the effects of NO

The NO − cGMP pathway

NO diffuses freely from its site of production (for example the endothelium) to its site of effect (for example the interior of the smooth muscle cell). Here NO activates the cytosolic, soluble guanylyl cyclase (sGC) which converts GTP to cGMP. The subsequent increase in cGMP concentration then mediates the effect of NO (for example smooth muscle relaxation) [94]. Also cGMP-independent effects of NO exist for example through the action of free radicals formed when NO reacts with oxygen and superoxide (O_2^-) [95].

NO from the endothelium: vasodilation

The endothelium is constantly exposed to the mechanical stress caused by the flow of blood. This so-called shear stress activates eNOS through direct phosphorylation and through an increase in endothelial cell Ca^{2+} concentration (which activates eNOS by binding to calmodulin) [96-100]. Also, prolonged shear stress on the endothelium over several hours leads to an up-regulation of eNOS expression [96]. Besides shear stress, eNOS is activated by different physiological factors such as growth factors (e.g. VEGF) and hormones (e.g. bradykinin, endothelin-1) [82, 101].

NO diffuses from the endothelium to the smooth muscle cells in the vessel wall and activates sGC by binding to its heme, leading to an increase in intracellular cGMP. The increased cGMP level leads to decreased interaction between the myosin and actin elements of the smooth muscle contractile apparatus by decreasing intracellular Ca^{2+} levels, which causes dephosphorylation of myosin and by making the contractile apparatus less sensitive to Ca^{2+} [84, 102]. The resulting smooth muscle relaxation leads to vessel vasodilation. Hereby, NO exerts a continuous vasodilator tone, thus lowering systemic blood pressure [103]. In addition, the vasodilation caused by NO increases blood flow in certain tissues such as the heart, brain, kidney, lung and the skeletal muscle at rest [104-107] and during exercise [108].

NO from the endothelium: additional effects

Besides vasodilation, NO synthesized by eNOS in the endothelium exerts additional effects influencing platelet, leukocyte and endothelial cell function.

Endothelium-derived NO inhibits platelet aggregation and adhesion by affecting both platelets and neighbouring endothelial cells. NO thereby prevents thrombus formation and promotes the free flow of blood [109, 110].

The actions of eNOS derived NO during inflammation appear to be two-fold. On the one hand, mediators of inflammation, such as bradykinin and histamine, cause vasodilation by stimulating NO synthesis. On the other, NO acts anti-inflammatorily by preventing leukocyte recruitment, adhesion, transmigration and secretion [111, 112], by maintaining vessel wall integrity thus preventing oedema formation, by regulating mast cell activity and by inhibiting platelet-mediated inflammatory response (adhesion, aggregation and release of inflammatory mediators such as serotonin, thromboxane and lipoxins) [113].

In addition, eNOS plays a role in free radical chemistry both in health and disease. In the healthy situation stimulation of the NO – cGMP pathway by shear stress leads to production of the free radical scavenging enzyme superoxide dismutase (SOD). SOD is the most efficient scavenger of the tissue-damaging free radical superoxide (O_2^-) and by stimulating SOD-production and O_2^- scavenging NO contributes to vessel wall protection. However, in hypertension, hypercholesterolemia, diabetes and ischemia so-called uncoupling of NOS has been identified. During these conditions the enzyme NADPH oxidase is stimulated to synthesize O_2^- and other free radicals which oxidise the eNOS co-factor BH_4 to dihydrobiopterin (BH_2). With BH_2 instead of BH_4 as a co-factor eNOS shifts (uncoupling of NOS) and starts producing O_2^- instead of NO, thus causing tissue damage [96].

Finally endothelial NO inhibits smooth muscle cell proliferation (decreased inhibition at arteriosclerosis leading to smooth muscle hyperplasia) but stimulates angiogenesis mediated by VEGF [114, 115].

NO in the nervous system

In the nervous system NO is mainly produced by nNOS, and here acts as a neurotransmitter and neuromodulator. In the central nervous system, short term effects of excitatory amino acids, pain perception, thermoregulation, appetite and sleep control as well as long term effects such as brain development, learning and memory (through so-called long term potentiations) are at least in part mediated by NO. In the brain NO is also thought to regulate blood flow and possibly integrate neuronal functions with blood flow [92, 116, 117]. In the peripheral nervous system, NO has been seen to be of importance in the enteric nervous system, the genito-urinary tract and in the respiratory tract through so-called nitrergic neurotransmission (one type of non-adrenergic non-cholinergic, NANC transmission). NO here has effects such as smooth muscle relaxation of the trachea, receptive relaxation of the stomach, relaxation of sphincters in the gastro-intestinal tract and penile erection [92, 117, 118].

NO in host defence

Early evidence showed that NO produced at high concentrations by the induction of iNOS in macrophages could exert toxic effects on pancreatic island cells [119] and protozoa [120]. During host-defence, iNOS in cell types such as neutrophils and macrophages is activated by cytokines and bacterial lipopolysaccharides (endotoxins) to produce NO at high concentrations. NO reacts with concurrently generated O_2^- to form peroxynitrite ($ONOO^-$) and other free radicals, which have a toxic and static effect on bacteria, viruses, protozoa, helminths or fungi [121-123]. It is now evident that iNOS can be induced in many other cell types, as originally described in blood vessels [124, 125].

NO binding to Hb. NO_2^- and NO_3^- as end products of NO metabolism

Already at the beginning of the 20th century, studies showed that hemoglobin (Hb) binds NO [126]. Later Hb was used as a blocker of the biological effects of NO in vitro [127] and after the discovery of NO in humans, Hb and especially oxygenated Hb (oxy-Hb) were recognised as being very potent scavengers of NO in vivo. In whole-blood oxy-Hb reacts with NO to form met-Hb (the oxidised form of oxy-Hb) and nitrate (NO_3^-) [128, 129]. In tissues, NO is oxidised to nitrite (NO_2^-), and further to NO_3^- in the presence of haem proteins. NO_3^- and NO_2^- have thus been regarded as end products of NO metabolism and are used as markers of NO production and NOS activity [130, 131].

Non-enzymatic NO production. NO_2^- and other endogenous NO donors

Before the discovery that the endothelium synthesizes NO, the in vitro formation of NO from NO_2^- was described. Murad and co-workers showed that NO_2^- and different "nitrovasodilators" released NO and hereby stimulated cGMP in a similar fashion as authentic NO gas [127].

The formation of NO from NO_2^- has now earned revived recognition as it has been shown to occur in vivo. NO is formed non-enzymatically from NO_2^- in a multi-step reaction with nitrous acid (HNO_2) as an intermediate **(formulas 1-3)**.

$$NO_2^- + H^+ \rightarrow HNO_2 \quad (1)$$
$$2HNO_2 \rightarrow N_2O_3 + H_2O \quad (2)$$
$$N_2O_3 \rightarrow NO + NO_2 \quad (3)$$

The reaction is greatly enhanced by acidity and reducing agents and its occurrence was first described in the gut by Benjamin and Lundberg independently [132, 133]. NO_2^- in the gut is derived either directly from dietary sources or from dietary NO_3^-, which in the mouth is reduced to NO_2^- by commensal bacteria [134, 135]. Non-enzymatic formation of NO from NO_2^- has also been described on the skin [136], in the urine [137] and in blood [135, 138]. NO thus formed is thought to participate in host defence and to stimulate blood flow.

In the blood, NO_2^- is no longer thought to be only an end product of NO metabolism but rather a storage form for NO and a potential endogenous NO donor. NO formation from NO_2^- in the blood leads to the release of NO distant from its first site of production during special conditions such as ischemia [138, 139]. Different routes of non-enzymatic NO synthesis from NO_2^- in blood have been described. As illustrated above, the reaction occurs spontaneously under acidic conditions, which is common during hypoxia. Also both xanthine oxidase and deoxygenated Hb have been seen to convert NO_2^- to NO under hypoxia. The released NO could promote blood flow during hypoxia in for example the ischemic myocardium [138, 139].

NO has been found to react with different compounds in blood. For example, NO forms adducts with thiols leading to nitrosylation of Hb. Through this process, the nitrosothiol S-nitroso-Hb (SNO-Hb) is formed [140-147]. Other examples of naturally occurring nitrosothiols are S-nitroso-albumin, S-nitroso-cysteine and S-nitroso-glutathione. These so-called SNOs have the general formula RSNO where R is an amino acid, polypeptide or protein [145, 146]. Furthermore, NO binds to heme in deoxygenated Hb forming NO-Hb [142]. Like NO_2^-, nitrosothiols and NO-Hb are thought to act as storage forms of NO and might be endogenous NO donors.

Alfred Nobel

Nitroglycerin, being very unstable, initially caused many fatal explosions and its inventor Sobrero had his face badly scarred after an accident. In 1850, the Swedish chemist Alfred Nobel visited the Pelouze laboratory-school in Paris and found Sobrero's invention very interesting. Nobel instantly realized the commercial potential of nitroglycerin and, after returning to Stockholm, together with his father Immanuel pursued studies on how to stabilize it. After several years of experiments and a terrible explosion that killed his younger brother Emil, Nobel was able to stabilize nitroglycerin with diatomaceous earth (kieselguhr). The combination was named Dynamite and became a large commercial success [79, 148].

Nobel spent his last days alone with his maid in San Remo, Italy. He suffered from angina pectoris but he was very reluctant to take the nitroglycerin that his doctor prescribed. Nobel died in 1896 and in accordance with his last will the bulk of his fortune was set aside to establish the Nobel Prizes. These were handed out for the first time in 1901 and in 1905 the French pacifist Bertha von Suttner received the Nobel peace prize. In the 1870s von Suttner (at that time named Kinsky) worked a short time for Nobel as "secretary and supervisor of household" and they remained friends by correspondence long after. She had a great influence on Nobel, inspiring him to become a pacifist and to come up with the idea of a peace prize [79, 148].

In 1998 Furchgott, Ignarro and Murad shared the Nobel Prize in physiology or medicine "for their discoveries concerning nitric oxide as a signalling molecule in the cardiovascular system".

3. Pharmacology of NO

NOS inhibitors

To inhibit NOS and NO synthesis, analogues of L-Arg have been utilized. These analogues inhibit NO synthesis by competing with L-Arg as a substrate. The first example was in studies on the endothelium using N^G-methyl-L-Arg (L-NMMA), where the guanidino moiety of L-Arg is substituted with a methyl group [88, 89]. Importantly, L-NMMA was used in many of the first findings on the physiological and pathophysiological roles of NO [149]. N^G-nitro-L-Arg methyl ester (L-NAME), where a NO_2-group has been added to the guanidino-nitrogen and where the α-carboxyl group has been esterified, is another example of an analogue to L-Arg acting as a NOS inhibitor. Subsequently, many additional L-Arg analogues have been developed aiming towards selective inhibition of the different isoforms of NOS [150, 151].

Additional examples of inhibitors of NOS are: L-citrulline analogues and imidazoles (both bind to the haem group of NOS), guanidines and isothioureas (both compete with L-Arg), indazoles and imidazoles (both compete with L-Arg and BH_4) and also BH_4 analogues [150, 151].

L-NMMA and L-NAME are fairly non-selective inhibitors of NOS. An example of a selective nNOS inhibitor is 7-nitroindazole (7-NI) [152] and examples of selective iNOS inhibitors are aminoguanidine and the isothiourea W1400 [150, 151].

NO donors

As NO accounts for many positive effects in the body, different ways of administering NO have been invented. Administration of NO as a gas can be performed by inhalation and NO is therefore used to treat pulmonary hypertension and respiratory distress syndrome (RDS) [153]. Administration of high concentrations of gaseous NO through inhalation is however limited by the formation of toxic concentrations of the gas nitrogen dioxide (NO_2). Furthermore, the inhaled NO reaches other tissues than the lung with difficulty due to the scavenging effect of Hb. Therefore different NO donors have been developed to release NO at the desired location. Long before the discovery of NO as a biological mediator, the NO donors amyl nitrite [154] and nitroglycerin [155] were used to treat angina pectoris. In the last couple of decades a multitude of additional NO donors have been developed aiming towards a controlled and targeted NO release. Some donors release NO spontaneously whereas others require previous metabolic transformation. Below follows a listing of a few of the most important NO donors.

Nitroglycerin, as do other organic nitrates, requires biotransformation and just recently, 140 years after its discovery, this has been shown to occur by mitochondrial aldehyde dehydrogenase [156]. NO thus released relaxes vessels, thereby increasing coronary flow and decreasing both pre- and afterload. Also, nitrates inhibit platelet aggregation. Repeated therapy with nitroglycerin leads to therapy resistance [157].

Morpholinosydnonimine (SIN-1), a sydnonimine is thiol independent, does not cause tolerance, and liberates NO spontaneously. As a consequence, SIN-1 causes venous and coronary vasodilation and also inhibits platelet aggregation [158, 159].

Nitrosothiols such as S-nitroso-N-acetylpenicillamine (SNAP) and S-nitroso-glutathione (GSNO) are commonly called SNOs and release NO when in contact with ultraviolet (UV) light, heat or trace amounts of copper. They thus display effects similar to those of NO [160]. As previously mentioned, endogenous SNOs such as GSNO, S-nitroso-albumin (SNO-alb), S-nitroso-cysteine (SNO-cysteine) and S-nitroso-Hb (SNO-Hb) are naturally occurring intermediates of endogenous NO metabolism [144].

Diazeniumdiolates, also called NONOates, contain a $[N(O)NO]^-$ group and release NO spontaneously without prior biotransformation [161, 162].

Sodium nitroprusside (SNP) releases NO when exposed to light or after contact with reducing agents. SNP exhibits powerful vasodilator effects but also contains and releases cyanide, which limits dosage [163].

The use of NO donors has proven beneficial in cardiovascular disease (treatment and prevention of myocardial ischemia, heart failure and hypertension) and could prove useful in the treatment of neurological disorders, inflammatory conditions and infections [164].

4. The microcirculation

In flap physiology and pathophysiology the microcirculation and regulation of blood flow in tissues such as skin and fat is important. In the microcirculation the endothelium holds a central position.

The endothelium

The endothelium consists of a single layer of approximately 10^{13} cells lining the inside of all blood vessels and became the centre of attention due to the discovery of prostacyclin [165], and even more so after the discovery of NO as an endogenous vasodilator [81, 85-87]. From supposedly only being an inert layer separating the blood and plasma from the interstitium, the endothelium was soon found to influence vascular tone, blood clotting, angiogenesis, inflammatory response and leukocyte activity, which in part has been described above.

Endothelium-derived vasoactive substances

Besides NO, several other vasoactive substances are also released from the endothelium.

Prostacyclin (prostaglandin I_2, PGI_2), discovered by Vane and co-workers in 1976 [165], is a potent vasodilator and antithrombotic agent acting on the smooth muscles in the vessel wall and on platelets in the vessel lumen. The synthesis of PGI_2 is initiated when phospholipase A_2 is activated by laminar shear stress to the vessel wall. phospholipase A_2 converts phospholipids from the endothelial membrane to arachidonic acid, which is then converted to prostaglandin H_2 (PGH_2) by the enzyme cyclooxygenase (COX). PGH_2 is subsequently converted to prostacyclin by the enzyme prostacyclin synthase. Prostacyclin acts on smooth muscle cells and platelets causing smooth muscle relaxation, vasodilation and inhibition of platelet aggregation through the adenylate cyclase—cyclic adenosine monophosphate (cAMP) system [166].

Besides PGI_2 and NO, a third endothelial derived vasodilator response has been identified. Here the smooth muscle cells are hyperpolarised and subsequently relaxed by a so-called endothelium-derived hyperpolarising factor (EDHF). No sole molecule or mechanism has been identified as EDHF but potassium ions (K^+), arachidonic acid metabolites from the epoxygenase pathway, gap junctions and hydrogen peroxide (H_2O_2) have all been suggested [167, 168].

Endothelin-1 (ET-1), which is synthesized upon vessel wall stretch and decreased sheer stress, acts on vascular smooth muscle cells, activating the enzyme phospholipase C. Through the second messengers inositol triphosphate (IP_3) and diacylglycerol (DAG) the intracellular Ca^{2+} concentration is increased leading to smooth muscle contraction and vasoconstriction. However, ET-1 also acts on the endothelium, stimulating the release of NO and prostacyclin, thus promoting vascular relaxation. In addition, NO inhibits ET-1 synthesis. ET-1 is thus, together with NO and prostacyclin, involved in a complex interplay regulating vascular tone, blood pressure and blood flow. ET-1 also has effects in the heart, kidney, lung and brain [169, 170].

Control of clotting and coagulation

Haemostasis with the prevention of thrombus formation is largely regulated by the endothelium through the synthesis of different substances. Platelet clotting is prevented by NO, prostacyclin, prostaglandin E_2 and endothelial heparan sulphate. The substances thrombomodulin (protein C activator), surface heparan sulphate (binds antithrombin III) and tissue factor pathway inhibitor (TFPI) inhibit coagulation, whereas tissue plasminogen activator (tPA) stimulates fibrinolysis. Under pathological conditions, the antithrombotic mechanisms are disrupted and in addition the endothelium releases prothrombotic substances such as the von Willebrand factor (vWF), which stimulates coagulation, and plasminogen activator inhibitor (PAI-1), which inhibits fibrinolysis [171, 172].

Endothelial effects on leukocytes

Under physiological conditions, the endothelium inhibits leukocyte activation by the release of substances such as NO [173]. During inflammation, seen for example during ischemia, the endothelium produces cytokines and expresses adhesion molecules that activate leukocytes [174] as will be discussed below.

5. Regulation of blood flow in skin and subcutaneous tissue

Skin

The skin is the largest organ of the body measuring approximately 1.5-2 m^2 in an adult (Mostellar method: skin area in m^2=square root of (height (cm) x weight (kg)/3600) [175]. The functions of the skin are diverse and include waterproofing, thermoregulation, barrier towards infection and control of blood pressure. The blood flow to the skin varies from 250 ml/min at rest to 7-8 l/min during heavy exercise or hyperthermia. This indicates that the skin is a well vascularized organ and that the blood vessels of the skin can undergo great changes in diameter [176].

Regulation of skin blood flow is achieved both through nervous control and through local humoral control. The nervous control consists of reflexes receiving information from baroreceptors and from thermal receptors centrally (hypothalamus) and peripherally (skin). The efference to the blood vessels is sympathetic and contains both vasodilator and vasoconstrictor signals. The reflexes are modulated centrally by factors such as training, fever, hydration and menstrual cycle [176].

Subcutaneous (adipose) tissue

Blood flow to the adipose tissue is mainly regulated to serve storage and release of its components. Thus, the blood flow increases during prolonged exercise and fast to liberate free fatty acids. After food intake, the flow also increases in order to promote the storage of triglycerides [177, 178]. In addition, orthostatic and thermoregulatory reflexes have been seen to influence the adipose blood flow [178]. NO is though to mediate a basal vasodilating tone whereas changes in blood flow probably are accomplished through sympathetic vasodilator (for example after meal intake) and vasoconstrictor activity [177].

6. Ischemia and reperfusion injury

Tissue damage due to ischemia or ischemia followed by reperfusion occurs in a number of clinical settings. Ischemia occurs when blood flow to the tissue is interrupted and if the blood flow later is re-established, reperfusion follows. In the heart, occlusion of a coronary vessel leads to myocardial ischemia and if the occlusion is dissolved through thrombolysis, the myocardium is reperfused. The same scenario occurs during thrombosis followed by thrombolysis in the lower extremity, brain, kidney or intestine [179-183].

In reconstructive surgery free flaps are first submitted to total ischemia as the circulation is cut at flap harvest. When blood flow has been re-established through vascular anastomoses, reperfusion follows. Also pedicled flaps may be subjected to ischemia. However, this ischemia is usually only relative and increases towards the distal end, the tip of the flap [184-187]. This distal ischemia is mainly seen in random pedicled flaps but at times also distally in axial pedicled flaps and distally in free flaps as the blood supply here is often of a random character. When partial flap failure occurs the distal flap tissue undergoing necrosis is separated from the surviving flap tissue by a hyperemic border zone [184].

It was established early on that ischemia in itself is tissue-destructive. Later evidence paradoxically showed that reperfusion with the re-establishment of blood flow and reintroduction of oxygen leads to even further destruction. The events taking place during ischemia and reperfusion are complex and a plethora of cells and mediators are involved. The mechanisms differ slightly between tissues but some main events are the same.

7. Ischemia

During ischemia, the oxygen supply in the tissue decreases, leading to decreased cellular oxidative phosphorylation and decreased synthesis of the energy-rich phosphates such as adenosine triphosphate (ATP). ATP dependent processes, including membrane bound ion pumps are halted, resulting in a cellular influx of calcium, sodium and water causing cellular hyperpolarization and swelling. Due to lack of oxygen, the metabolism becomes anaerobic, leading to acidosis [188-191].

Furthermore, free radical chemistry is initiated during ischemia. Mitochondrial scavengers of O_2^- are depleted and xanthine dehydrogenase is converted to xanthine oxidase (the two interconvertible forms of the enzyme with the generic name xanthine oxidoreductase, originally called xanthine oxidase). As described below this leads to the formation of free radicals when oxygen is reintroduced at reperfusion [192-194].

Also, proinflammatory cytokines such as interleukins and tumour necrosis factor alpha (TNFα) are released from the ischemic tissue. These cytokines increase local blood flow and up-regulate the expression of surface adhesion molecules (membrane bound glycoproteins) on endothelial cells, leukocytes and platelets. At the same time, synthesis of protective factors from the endothelium such as NO, thrombomodulin and prostacyclin are decreased [191, 195-201]. As a result, accumulation and activation of leukocytes and platelets is promoted, especially during reperfusion.

Ischemia is thus damaging in itself and if sustained, leads to tissue necrosis. The ischemic insult also causes a state prone to further destruction once reperfusion occurs.

8. Reperfusion

Leukocyte – endothelial cell interaction

As described above, ischemia causes the release of cytokines and expression of cell surface adhesion molecules. At reperfusion, this causes an inflammatory response involving the endothelium and leukocytes (such as neutrophil granulocytes). In a process ultimately leading to leukocyte extravasation and activation, leukocytes and endothelium interact as follows: In the first step, leukocyte rolling or loose adhesion, the leukocytes bind loosely to the endothelium through the coupling of the selectin family of adhesion molecules. The most important molecules are E- and P-selectin on the endothelial cells and L-selectin on the leukocytes. The rolling brings the leukocytes in closer proximity to the endothelium and in contact with chemoattractants such as leukotriene B_4 (LTB_4), platelet activating factor (PAF), and complement C5a. These chemoattractants facilitate the next step of leukocyte-endothelial cell interaction called firm adhesion. The leukocytes now bind more tightly to the endothelium through the coupling of receptors from the integrin family of adhesion molecules. CD11/18 on the leukocytes thus binds ICAM-1 on the endothelium. During the third and final step the leukocytes transmigrate between the endothelial cells into the interstitium using diapedesis [195-199]. Once in the interstitium, the leukocytes release a number of substances such as elastases, proteases and free radicals causing tissue damage and promoting the inflammatory response [191, 202, 203].

Complement factors

The complement system consists of a row of plasma proteins normally involved in host defence. During ischemia and reperfusion the system is activated, thus increasing the inflammatory response and contributing to tissue damage. Some complement factors attract and activate leukocytes (C3a and C5a) while others promote leukocyte-endothelium interaction by stimulating selectin and integrin synthesis and activity. The complement MAC (membrane attack complex) causes cell lysis and finally, some complements cause vasoconstriction thus decreasing blood flow [204, 205].

Free radicals

During reperfusion oxygen takes on a malicious role as it suddenly is converted into the free radical O_2^-. The conversion mainly occurs enzymatically by xanthine oxidase and mitochondrial enzymes in the damaged tissue and by NADPH oxidase in leukocytes. Also non-enzymatic formation is seen through the reaction between oxygen and iron released from haem and cytochromes upon tissue destruction [206].

The two final reactions during the breakdown of purines (adenosine monophosphate, AMP and guanosine monophosphate, GMP) are the conversion of hypoxanthine to xanthine and then xanthine to urate, the end product of purine metabolism. The conversion is normally catalysed by the enzyme xanthine dehydrogenase, which at the same time reduces $NADP^+$ to NADPH. During ischemia, however, xanthine dehydrogenase is converted to xanthine oxidase, which is unable to utilise $NADP^+$ as an electron acceptor. Instead xanthine oxidase uses oxygen (reintroduced at reperfusion) which it reduces to the highly reactive free radical O_2^- [192, 193].

Under normal conditions the mitochondria reduce oxygen to water through the electron transport chain (linked to oxidative phosphorylation). At the same time small amounts of normally formed O_2^- are scavenged by SOD and glutathione peroxidase. During ischemia the electron transport chain, SOD and glutathione peroxidase are impaired. When oxygen is reintroduced at reperfusion it is converted to O_2^- instead of to water, O_2^- is no longer scavenged and a massive build up in O_2^- occurs [194].

At reperfusion leukocytes, mainly neutrophil granulocytes and macrophages are attracted to the tissue as described above. Upon activation they produce O_2^- from oxygen by the enzyme NADPH oxidase [207-209].

As described earlier, NOS can also take part in free radical formation. This occurs through the uncoupling of NOS, which thus converted oxygen to O_2^- instead of synthesizing NO.

O_2^- formed through the above described routes is subsequently converted into other free radicals such as hydroxyl radical (OH), hypochlorous acid (HOCl), H_2O_2 and, after the reaction with NO, $ONOO^-$ [210, 211].

The effects of free radicals are deleterious to the tissue and cause protein denaturation (e.g. destruction of enzymes and membrane channels), direct DNA damage, cytoskeletal destruction and cell membrane disruption. The latter effect occurs both through direct lipid peroxidation and through the activation of phospholipase A_2 [206, 210]. Phospholipase A_2 hydrolyses membrane phospholipids, thus forming arachidonic acid, which via intermediary hydroperoxy acids is converted to thromboxane A_2 (TXA_2) and LTB_4 by cycloxygenases (COX) and lipoxygenases. TXA_2 and LTB_4 in turn are proinflammatory by attracting and activating platelets and leukocytes and by increasing microvascular leakage [212, 213].

Platelets
Also platelets have been shown to take part in ischemia and reperfusion injury through interactions with the endothelium, leukocytes and other platelets. The interaction with the endothelium shows similarities to the leukocyte-endothelial cell interaction described above. Platelets thus first roll along the endothelium and then firmly adhere. During firm adhesion, the platelets release substances, for example platelet activating factor (PAF), serotonin and TXA_2 that modify the endothelium and attract and activate leukocytes and other platelets. As a result, additional chemoattractants are released and adhesion molecules are expressed thus facilitating leukocyte-endothelium, platelet-endothelium and platelet-leukocyte contact and interaction. Hereby leukocytes not only adhere to the endothelium, but also to the already adherent platelets leading to the formation of larger and larger cell-conglomerates in the vessel lumen.

During ischemia-reperfusion, platelets thus are both proinflammatory and also cause mechanical obstruction of blood flow by creating platelet-leukocyte aggregates [191, 200, 214] .

The no-reflow phenomenon
Damage to capillaries and postcapillary venules during ischemia leads to a situation called the “no-reflow”. This event is characterized by the clogging of blood vessels with platelets and leukocytes and also by the compression of the vessel lumen due to swelling of the endothelium and interstitium. Thus, even though adequate tissue blood flow might have been established during reperfusion, the microcirculation is unable to receive it [191, 214, 215].

9. The role of NO during ischemia and reperfusion

NO has been found to have a dual role during ischemia and reperfusion. On the one hand it seem to be tissue protective and on the other it seems to be tissue destructive [216].

Tissue protection. The good NO
As mentioned above, NO has a number of effects that are beneficial during ischemia and reperfusion. NO from the endothelium dilates blood vessels and increases blood flow thus preventing ischemia. NO also prevents leukocyte and platelet adhesion and aggregation, thereby preventing inflammation and thrombus formation [113]. Furthermore, NO acts as a scavenger of free radicals [217] and also stimulates endothelial cell regeneration [115, 218]. Lack of NO could therefore be a factor in the no-reflow phenomenon.

These beneficial effects of NO are probably achieved by a continuous release of small amounts (picomoles) of NO from constitutive eNOS and nNOS [217, 219]. Ischemia and reperfusion lead to impaired constitutive NO production due to endothelial dysfunction and decreased shear stress stimulus on the endothelium [191, 201]. In addition the bioactivity of constitutive NO could be reduced [220, 221].

Tissue destruction. The bad NO

During ischemia and reperfusion, iNOS mainly in leukocytes, is induced by cytokines, endotoxins and lipid mediators leading to the production of NO at high concentrations (nanomoles). Also endothelial cells, smooth muscle cells and fibroblasts are capable of synthesizing NO by iNOS [125, 222]. NO at high concentrations has been shown to cause tissue damage in itself by blocking mitochondrial electron transport [223] and by causing DNA strand breaks [224]. In addition, high concentrations of NO lead to the formation of different free radicals. NO thus reacts with O_2^- to form $ONOO^-$ that reacts with hydrogen ions (H^+, abundant due to acidity) to form peroxynitrous acid (HOONO) which in turn dissociates into NO_2 and the highly reactive OH [217, 225].

Studies have also shown that iNOS can be beneficial and tissue protective [226, 227].

10. NO in flap ischemia and reperfusion

In recent years, research on the role of NO in flap tissue has arrived at conflicting results. Non-selective NOS inhibition has been shown to increase [228-231], decrease [232] or not affect [231, 233] flap survival. Unlike results from the authors (unpublished), L-Arg has proven beneficial to flap survival [234, 235], as have different NO donors [236-238]. Evidence from studies on iNOS knockout mice is conflicting, showing both increased and decreased injury after ischemia and reperfusion [239, 240].

AIMS OF THE THESIS

- To study the effects of flap ischemia on flap NOS activity and to correlate changes in NOS activity to changes in flap morphology

- To study the role of iNOS in flap survival by inhibition of its induction

- To study the role of constitutive NOS activity in flap blood flow control and flap survival by use of NOS inhibition

- To study the effect of topically applied NO on flap blood flow

MATERIALS AND METHODS

1. Animals

Ethical approval for the experiments was obtained from the Stockholm Committee for animal experimentation (diary no. N183/93, N176/96, N262/99, N200/03, N384/05).

All in vivo experiments in this thesis were performed on laboratory rats as they are of a convenient size, relatively calm, easy to handle and economical to maintain. Furthermore, rats are easily accessible for surgical intervention and also show many similarities to humans with regard to metabolic pathways and anatomical and physiological characteristics [241]. More specifically, the experiments utilized male albino Sprague Dawley rats, which is a widely accepted and widely studied research model used in most fields of biomedical research [242].

2. Anaesthesia, body temperature and hair removal

In **Papers I and II**, chloral hydrate was used for anaesthesia and analgesia. At the time of these experiments, chloral hydrate was widely used and popular for short surgical procedures in rodents and other small laboratory animals. We found the dose 400 mg/kg administered intraperitoneally adequate, as have others before us. In order to eliminate the risk of peritonitis and adynamic ileus, a low concentration solution (50 mg/ml in saline) was used [243, 244]. Intraperitoneal injection was chosen as this is a relatively easy and reliable route of administration.

In **Papers III and IV**, registration of blood flow and blood pressure after flap surgery was included in the experimental setup. Sodium pentobarbital, a very common anaesthetic for rodents, was here chosen as it causes only moderate cardiovascular depression while giving a stable anaesthesia for a long period of time [245]. During surgery and drug treatment, administration was performed through continuous intravenous infusion (20 mg/kg/h in saline) as this makes it easy to maintain an adequate depth of anaesthesia and analgesia and also results in a stable influence on blood pressure [243-245]. Intraperitoneal administration (40 mg/kg) was chosen for anaesthesia during hair removal and induction before surgery as it is effective within minutes [243, 244].

During anaesthesia and the postoperative wake-up period, care was taken to keep the rats' body temperature normal at 38±0.5°C, using a heating pad.

In order to achieve optimal conditions for laser Doppler perfusion analysis and later for flap survival determination, the hair of the designated flap area was removed using an electrical shaver followed by a hair-removing cream.

3. Experimental skin flap models

Two different random flap models on the rat's dorsum were used in the study, one with the base located cranially and one with the base located caudally.

Cranially based dorsal random skin flap model. Paper I

In 1965 McFarlane described a dorsal skin flap model in the rat [246]. Subsequently, it was further described with regard to anatomy and histology and became a frequently used model for studies on flap ischemia and survival [247-249]. The flap, which measures 2x7 cm, was used in **Paper I** and is relatively easy to raise as follows: First the flap is outlined along the rat's dorsum using a template, placing the tip where the two gluteus muscles meet in the midline and the base cranially. The flap is then cut out with a sharp pair of scissors on all sides except the cranial side which forms the base. Skin, subcutaneous tissue, panniculus carnosus and superficial muscle fascia are included and after having been raised, the flap is sutured back into its previous location. Due to its design, the flap subsequently develops a distal necrosis within one week, representing approximately 50% of the flap surface. However, we have noticed that this necrosis is only superficial and, if the flap is left for another week, the necrosis is shed and the flap survives, the reason probably being ingrowth of vessels from the sides and underlying tissue. The dorsal flap is a so-called "random" flap as it does not have a defined blood supply. Instead it relies on many small blood vessels entering at random.

Caudally based dorsal random skin flap model. Papers I-III

The dorsal McFarlane flap was subsequently modified by several researchers and in 1993 Hammond described a modification where the flap, still with the same dimensions and containing the same tissue layers, is based caudally and where the skin is sutured in the midline under the flap **(fig 1)** [250]. In this flap model, a clear demarcation line is evident within one day and a non-reversible, distal full-thickness necrosis is seen within three days **(fig 2)**. This flap model was used in **Papers I-III**.

Determination of flap survival
Papers I-III

To determine flap survival, a 2x7 cm piece of transparent plastic was put on each flap, and the demarcation line **(fig 2)** between viable and necrotic tissue was marked with a pen. The plastic was copied to paper in a copying machine, and the two different areas were then weighed separately on sensitive scales. In this manner, the percentage of surviving and necrotic flap area could be calculated.

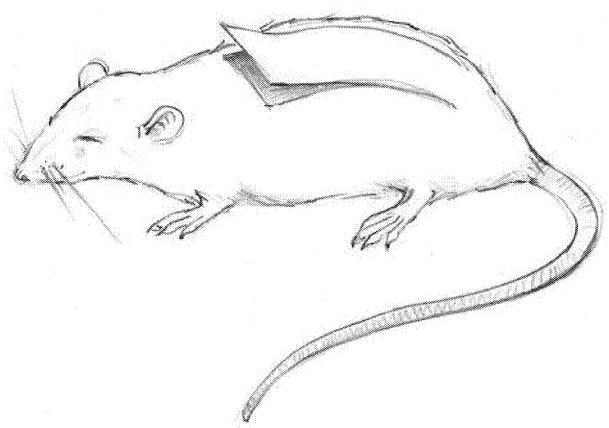

Figure 1. Caudally based random flap.

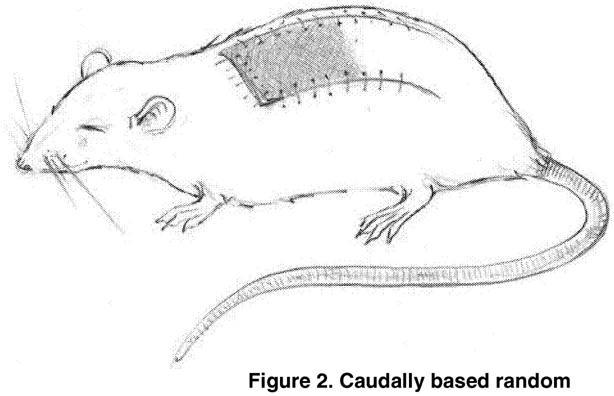

Figure 2. Caudally based random flap sutured in place and showing distal necrosis.

Ventral epigastric island skin flap model. Paper IV

In **Paper IV**, a previously described flap located on the rat's abdomen was used [251-253]. Unlike the two dorsal random flaps, this flap has a defined blood supply through the inferior superficial epigastric artery and is drained by the corresponding vein. The flap used measured 4x6 cm and was outlined using

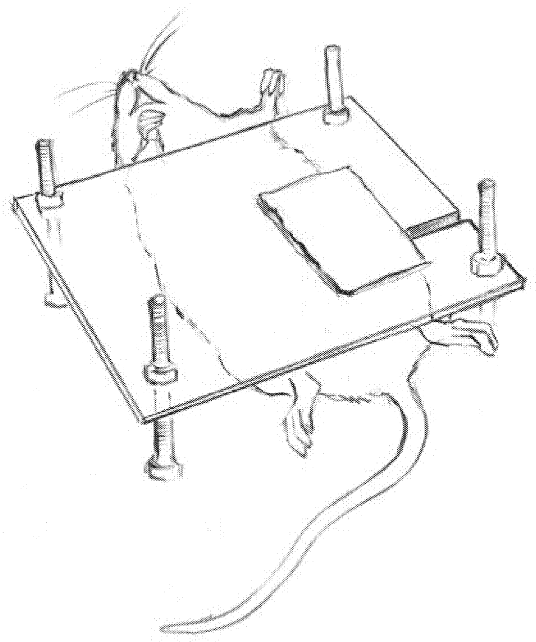

Figure 3. Ventral epigastric skin flap positioned on top of the hard plastic mount.

a template. The borders were cut out with a scalpel and the flap including the skin and subcutaneous tissue was raised from the underlying muscle fascia using a sharp pair of scissors. Care was taken to include the same amount of subcutaneous tissue each time and in doing so, the lateral arterial branch running in the subcutaneous fat was always included [252]. The nerve and vessel bundle was then freed under microscopic magnification using microsurgical instruments. The nerve was cut to mimic the conditions of a free flap and the artery and vein were carefully freed from one another. As the main vessels to the flap were freed, some minor vessels were cut so that the flap finally was supplied by its main artery and vein alone. It has been shown that these minor vessels probably are of less importance [254].

After having been raised, the flap was positioned flat on top of a mount of hard plastic just above the rat's body **(fig 3)**. The flap continued to maintain its blood supply via the vascular pedicle (artery and vein) reaching the flap through a hole in the hard plastic. This modification to the model renders it possible to measure blood flow both in the supplying artery (and vein) as well as in the surface of the flap without the interference of artefacts caused by breathing movements. The modification has not been described previously.

4. NOS quantification by citrulline assay. Papers I-III

In **Papers I-III**, NOS activity was measured using the so-called citrulline assay [255]. In this analysis, a small amount of radioactive L-arginine (L-[U-^{14}C] arginine) is added to the substrate, i.e. the flap tissue homogenate, which results in the formation of radioactive L-citrulline (L-[U-^{14}C] citrulline). As NOS converts L-arginine to L-citrulline during its synthesis of NO, the amount of radioactive L-citrulline denotes the activity of NOS. Briefly, tissues were homogenised in a buffer. After centrifugation the soluble fraction was added to tubes with a buffer containing among other things L-[U-^{14}C] arginine. Duplicate incubations at 37°C were performed both in the presence and the absence of either EGTA or EGTA plus L-NAME to determine the level of Ca^{2+} dependent and Ca^{2+} independent NOS activity. The reaction was terminated and the presence of L-[U-^{14}C] citrulline was determined by scintillation counting. The level of citrulline was expressed as picomoles per gram of tissue (wet weight) per minute.

5. Transmission electron microscopic studies. Paper I

Electron microscopic studies were performed in **Paper I** on caudally based flaps to examine changes in flap ultrastructure.

Thin samples from the proximal and distal part of the flaps and control skin were cut out using a scalpel. The samples were fixed in 2.5% phosphate-buffered glutaraldehyde for 7 days, post-fixed in 2% osmium tetroxide and stained *en bloc* with 1% uranyl acetate. After progressive dehydration in a graded series of ethanol washes, ending with propylene oxide, the samples were transferred to a 50/50 mix propylene oxide and Agar Resin where they remained for 24 hours at room temperature. This was followed by a change to 100% Agar Resin, in which the specimens were polymerised at 40°C for 24 hours and at 60°C for 48 hours. Semi-thin sections were cut, stained with toluidine blue and under a light microscope, sections suitable for electron microscopy were determined. Ultra-thin sections were cut out and mounted on copper grids, stained with uranyl acetate and lead citrate, and examined in a Carl Zeiss EM-109 transmission electron microscope (TEM) operating at 50 kV. The images were projected on to black-and-white film and, using standard dark-room procedure, the negatives were developed, their images transferred to photographic paper using an enlarger and the prints developed.

Signs of endothelial damage such as chromatin margination, cytoplasmic vacuolisation, disrupted membranes (blebbing), cellular swelling and endothelial loss were looked for [256, 257].

6. Blood pressure recordings. Papers III and IV

Systemic mean arterial blood pressure was measured through a cannula, which was placed in either the left superficial femoral artery in **Paper III**, or the left common carotid artery in **Paper IV**. The cannula was connected to a transducer (Statham, Puerto Rico) and the signal was amplified by a low-level DC amplifier (Grass Instruments, Quincy, MA, USA). The amplified signal was then digitalized in an AD-converter (Analogue-Digital) and recorded in a personal computer.

7. Blood flow study methods

Laser Doppler technique. Papers III and IV

Superficial capillary blood flow was measured in **Papers III and IV** using two different laser Doppler techniques. In **Paper III**, laser Doppler perfusion monitoring was used whereas in **Paper IV**, laser Doppler perfusion imaging was used. Laser Doppler technique utilises the phenomenon of Doppler shift to measure the movement of blood cells. A laser beam is transmitted 0.5-1 mm into the tissue where some of the laser light hits moving blood cells, thus changing its frequency, "Doppler shift". The reflected light is taken up by a receiver and by analyzing (1) the proportion of frequency shifted light to unshifted light and (2) the magnitude of the frequency shift of this light, the concentration and mean velocity of the moving blood cells are calculated respectively. The concentration of moving blood cells, multiplied by the mean

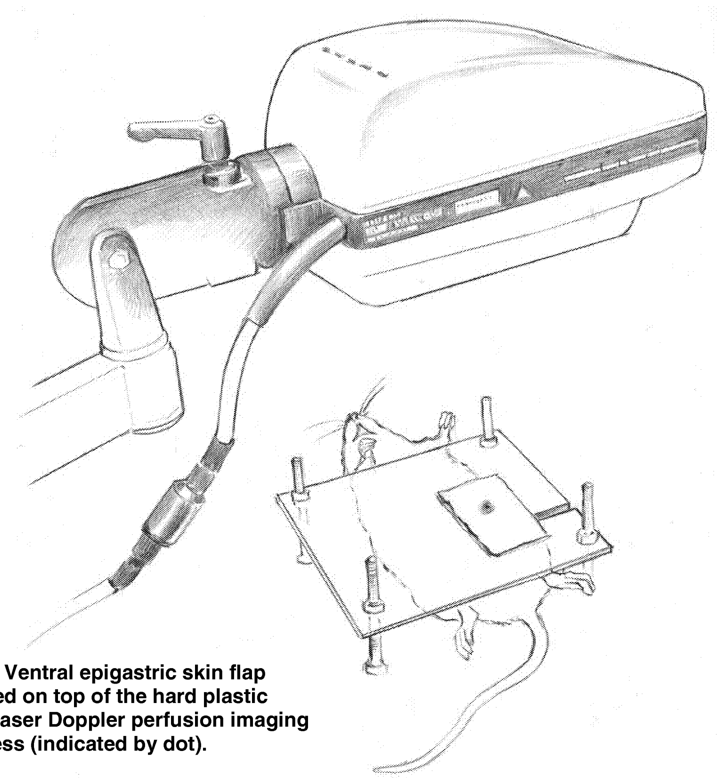

Figure 4. Ventral epigastric skin flap positioned on top of the hard plastic mount. Laser Doppler perfusion imaging in progress (indicated by dot).

velocity of these cells, is expressed as Perfusion Units (PU, earlier called "flux units"), which is the value displayed by the laser Doppler apparatus. PU are arbitrary values of perfusion but can, in the same type of tissue and using the same type of instruments be compared after calibrating the laser Doppler probe. To accomplish this, a liquid containing moving, light-scattering polystyrene microspheres whose motility is sustained by Brownian motion is used (Motility standard, Perimed, Stockholm, Sweden). No current laser Doppler instrument can present absolute perfusion values (e.g. ml/min/100 gram tissue) but a close correlation between PU and blood flow has been demonstrated [258, 259].

Laser Doppler perfusion monitoring. Paper III
For laser Doppler perfusion monitoring in **Paper III**, a laser Doppler flow meter and two identical laser Doppler probes were used (Perimed 4000 and two PF 408, Perimed). The probes were placed on the proximal and distal portion of the flaps or, in the controls, on intact skin. Measurement values (PU) were digitalized in an AD-converter (Perimed 472, Perimed) and continuously saved into a personal computer.

Laser Doppler perfusion imaging. Paper IV
In **Paper IV**, laser Doppler perfusion imaging technique (PIM II laser Doppler perfusion imager, Perimed) was used **(fig 4)**. In this procedure, a laser beam successively scans the tissue, generating a colour coded picture and a mean perfusion value. To optimize the time for taking an image and still measure all the tissue the step length is set to be the same as the size of the laser beam.

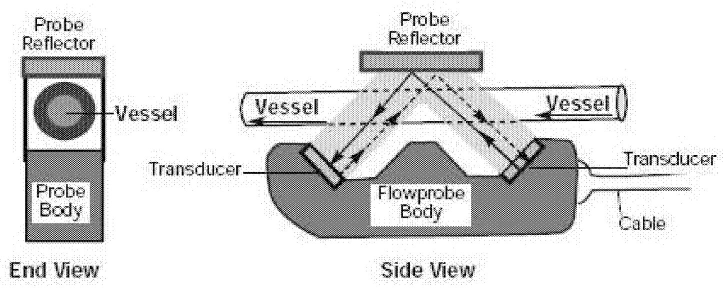

Figure 5. Transit-time ultrasound flow probe.
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Transit-time ultrasound flowmetry. Paper IV
In **Paper IV**, blood flow in the epigastric artery supplying the flap was measured using transit-time ultrasound technique. The system used was a 0.5 mm V flow probe connected to a T206 animal research flowmeter (Transonic Systems, Ithaca, NY, USA) and the data was collected at a sampling rate of 200 Hz. The probe contains two transducers, which send ultrasonic beams through the blood stream at oblique angles in opposite directions (**figs 5 and 6**). The ultrasonic beam will pass through the vessel faster with the direction of blood flow (downstream) than in the opposite direction (upstream). The "transit-time" for the ultrasonic beam is thus shorter in the downstream direction than in the upstream direction and, using this information, the blood flow can be calculated by the flowmeter and expressed in for example ml/min [260-263]. The signals from the flowmeter were digitalized in an AD-converter (Perimed 472, Perimed) and subsequently recorded and analysed in a personal computer using the Perisoft software (Perimed).

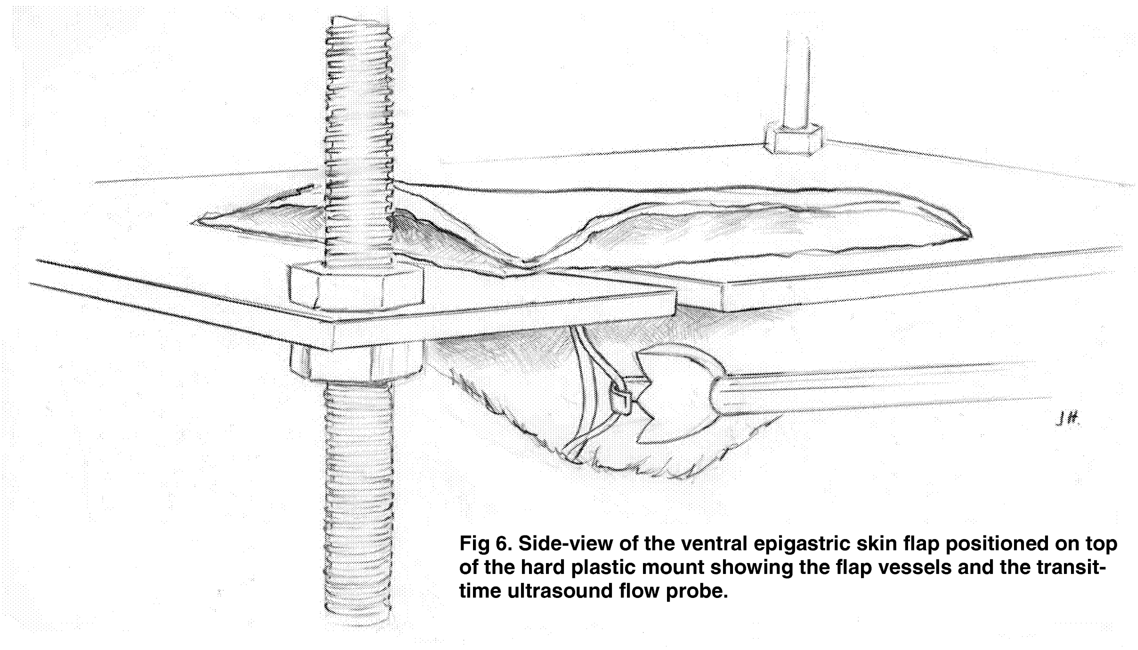

Fig 6. Side-view of the ventral epigastric skin flap positioned on top of the hard plastic mount showing the flap vessels and the transit-time ultrasound flow probe.

8. Measurement of NO and NO_2 formation

In **Paper IV**, NO and NO_2 generation from a cream containing acidified NO_2^- was measured by chemiluminescence technique (Monitor Labs 9840 Nitrogen Oxides Analyzer; Monitor Labs, Englewood, CO, USA). The NO generating cream was mixed and put into an air-tight chamber (volume 6.5 litres) sampled at 640 ml/min, withdrawn air being replaced by air free from nitrogen oxides. In the analyzer, NO reacts with ozone (O_3) to form excited state NO_2. The excited state NO_2 then returns to its lower energy level, at the same time emitting light, chemiluminescence. The amount of emitted light represents the NO concentration. The gas sample is passed through a catalytic converter at regular intervals, which converts NO_2 to NO. Through this procedure, a summated NO concentration called NO_x is obtained and the NO_2 concentration is calculated as NO_x minus NO.

9. Administration of treatment drugs and placebo. Paper II-IV

Treatment drugs and placebo were administered either intraperitoneally, intravenously or transdermally.

In **Paper II**, treatment with 1 mg/kg of dexamethasone in saline was performed by way of intraperitoneal injection. Intraperitoneal injection is relatively easy to perform and the dose 1 mg/kg has previously been shown to be adequate for iNOS inhibition [264-266].

In **Paper III**, the non-selective NOS inhibitor L-NAME was used. After mixing L-NAME in saline, administration was performed intravenously by a bolus dose of 100 mg/kg, followed by a continuous infusion at 10 mg/kg/hour. The dose 100 mg/kg has previously shown to be effective for the inhibition of NOS [267]. D-NAME (the inactive enantiomer of L-NAME) in saline and saline alone were used as controls. Intravenous administration was chosen to maximize distribution, thus increasing the chances for the drugs reaching the target tissue, the flap. Other ways of administration such as intraperitoneal, subcutaneous or intramuscular injection were not chosen as L-NAME, being a vasoconstrictor, could prevent uptake from these injection sites.

In **Paper IV** a cream for local NO administration to flap tissue was created by mixing two creams (50/50 mixture) containing equal concentrations of NO_2^- and vitamin C (ascorbic acid), to yield the final NO_2^- concentrations of 0.125%, 0.25%, 0.5%, 1.25% and 2.5%. Mixing was done on the flap surface to standardize the onset of NO generation, which starts upon contact between NO_2^- and vitamin C [268, 269]. Cream base alone and cream base acidified with vitamin C (final concentration 2.5%) were used as controls.

10. Statistical analysis. Papers I-IV

In **Paper I**, the one-way Analysis of Variance (ANOVA) was used to compare multiple independent means of Ca^{2+} dependent and Ca^{2+} independent NOS activity respectively. Post hoc pairwise comparisons between effects of different time-points after surgery on NOS activity were performed using the Tukey's honest significant difference (HSD) test.

In **Paper II**, two independent means of flap survival and NOS activity were analysed using the Student's t-test after assuming data to be approximately normally distributed, and Mann-Whitney U-test where no assumption regarding the distribution was made.

In **Paper III**, the two-way ANOVA was used to compare multiple dependent means of blood pressure and relative values of laser Doppler perfusion between L-NAME, D-NAME or saline treatment groups. Post hoc pairwise comparisons between effects of different time points after treatment start on blood pressure and perfusion were performed using the Dunnett's test. Student's t-test was used when comparing two independent means of flap survival and NOS activity.

In **Paper IV**, the one-way ANOVA with repeated measures design was used to compare multiple dependent means of NO, NO_2, blood pressure and supplying blood flow and to test for differences between independent treatment groups. The Tukey's HSD test was used post hoc to compare effects of two different concentrations on superficial blood flow.

All tests were two-sided and $p<0.05$ was regarded as statistically significant.

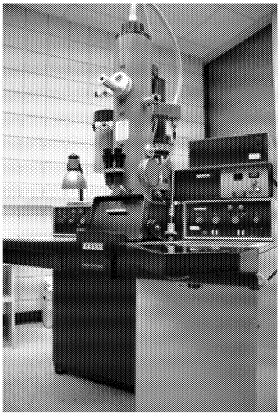

Fig 7. The Carl Zeiss electron microscope

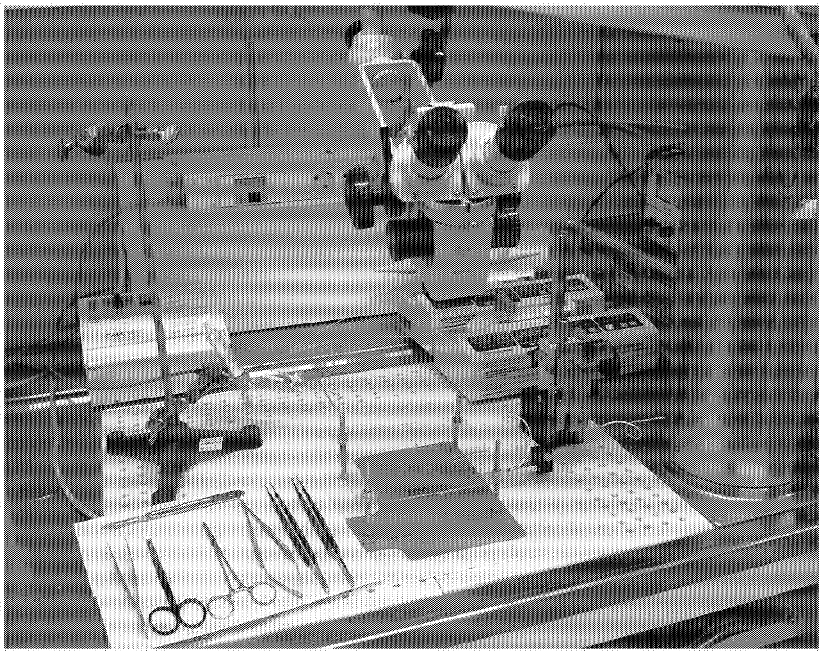

Fig 8. Laboratory set-up showing microscope, surgical instruments, infusion pumps for the administration of anaesthetic and treatment drugs, hard plastic mount for flap fixation, flowmetry probe, arterial pressure-transducer and heating pad.

RESULTS AND DISCUSSION

The results have been presented in detail in **Papers I-IV** and below follows a description of the most important results.

1. Endothelial damage and decreased constitutive NOS in flaps. Paper I

In control samples of intact skin, the presence of constitutive Ca^{2+} dependent NOS activity was found using citrulline assay. With increasing time after surgery this NOS activity drastically declined both in proximal and distal parts of dorsal random skin flaps **(fig 9)**. The decrease was most rapid in onset in the distal part of the flaps. Parallel to these findings, TEM studies showed signs of damage to blood vessels and endothelium as previously described by others [256, 257]. The changes were most pronounced in the distal part of the flaps where after only four hours signs of incipient endothelial damage were seen. These changes included cellular swelling, blebbing of cellular membranes, chromatin margination and cytoplasmic vacuolization **(fig 10)**.

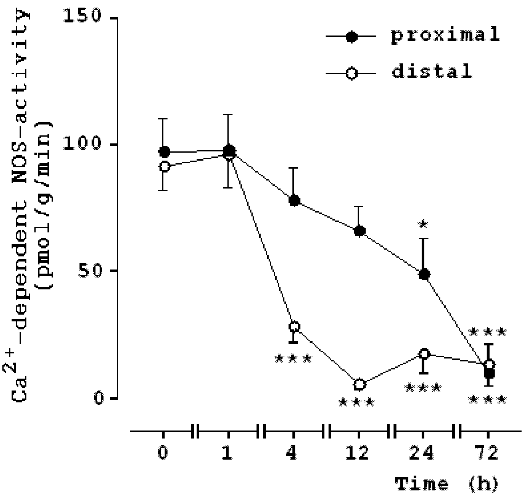

Fig 9. Decrease in constitutive NOS activity.

Subsequently the endothelial destruction accelerated and the distal part of flaps removed after 12 hours showed severe stasis and endothelial loss of contact with the basement membrane, allowing red blood cells to pass into the surrounding interstitium. It was not possible to investigate the distal part

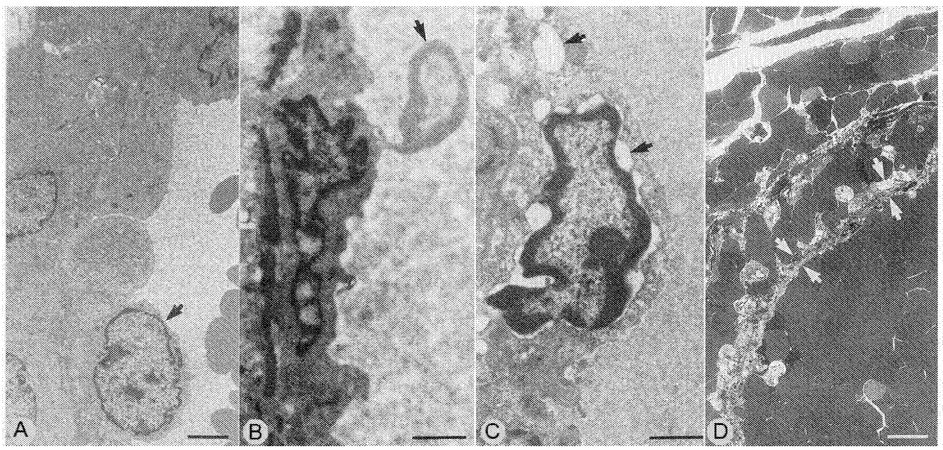

Fig 10. TEM pictures showing endothelial damage. Cellular swelling (A), blebbing (B), vacuolisation (C), endothelial loss of contact with basement membrane and severe stasis (D). Bar 1µm.

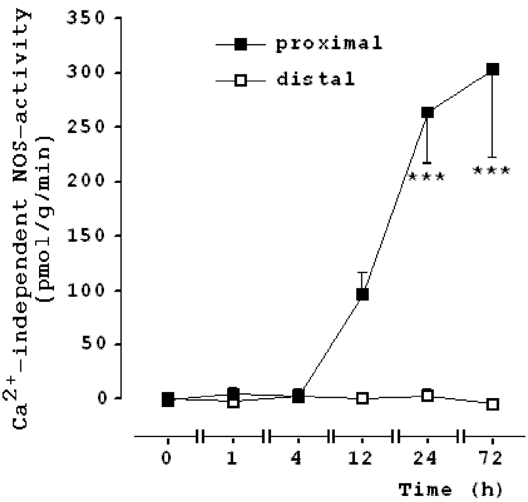

Fig 11. Induction of iNOS.

As the endothelium probably is the main source of constitutive NOS activity in skin flaps, the observed endothelial damage likely accounted for the decrease in constitutive NOS activity [191, 201].

of the flaps removed at 72 hours due to severe tissue damage. In the proximal part of the flaps the damage was not at all as apparent. However, in flaps removed at 72 hours the endothelium here showed cellular swelling and blebbed cell membranes. In addition occasional destruction of veins parallel to an accumulation and extravasation of leukocytes and aggregation of platelets as described below was seen.

2. Infiltration of leukocytes and induction of NOS in flaps. Paper I

Control samples of intact skin did not show any inducible Ca^{2+} independent NOS (iNOS) activity as measured by citrulline assay. With increasing time after surgery however, a prominent rise in iNOS was seen in both dorsal random skin flap models used in this thesis. The rise was most marked and very prominent in the proximal part of the most ischemic, caudally based flap model **(fig 11)**. Parallel to these findings infiltration of leukocytes, mainly neutrophil granulocytes and macrophages were observed using TEM **(fig 12)**. In addition aggregation of platelets was seen in the vessel lumen. The iNOS activity was probably derived from the leukocytes but the source could also be the platelets as well as endothelial cells and fibroblasts [125, 222].

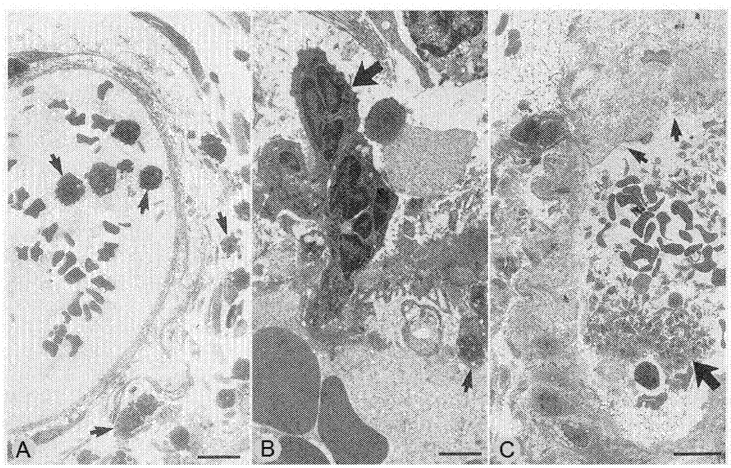

Fig 12. TEM pictures showing leukocyte accumulation (A) and immigrationg (B). Trombusformation and vessel wall destrucion (C). Bar 1µm.

3. Effect of dexamethasone on iNOS activity and flap survival. Paper II

Dexamethasone, given as a 1 mg/kg single dose intraperitoneally three hours prior to the surgery of dorsal, caudally based, random skin flaps, partially prevented induction of Ca^{2+} independent NOS activity (iNOS) in flaps and increased flap survival as compared to controls (**Paper II, figs 1 and 2**).

Studies have shown that dexamethasone inhibits iNOS activity by reducing iNOS gene transcription, mRNA stability and translation and also by increasing iNOS protein degradation [270, 271].

Inhibition of iNOS using dexamethasone has been shown to preserve endothelial cells [272] and to be tissue protective during ischemia and reperfusion in the heart [273], the brain [274], the kidney [275], the colon [265] and skeletal muscle [276]. This protective effect of dexamethasone, as well as the one seen in **Paper II**, could be explained by a reduction of the negative effects of iNOS. A high concentration of NO, formed by iNOS upon induction, has proven to be tissue damaging both in itself and, more importantly, through the formation of tissue damaging free radicals such as $ONOO^-$ and OH [217, 225]. The tissue protective effect of dexamethasone and other glucocorticoids could also be attributed to additional anti-inflammatory actions such as the preservation of vascular integrity, reduced edema [276, 277], inhibited accumulation and activation of leukocytes [275, 277, 278] and reduced formation of pro-inflammatory cytokines, prostaglandins and leukotrienes [275, 278-281]. Glucocorticoids have also been seen to be directly tissue protective, inhibiting membrane lipid peroxidation by scavenging free radicals and stabilising cellular membranes [277]. Furthermore, positive effects on muscle recovery after ischemia and reperfusion have been demonstrated [277].

In plastic and reconstructive surgery, especially in the head-and-neck region, corticosteroids are often used as they are considered safe and are supposed to reduce edema [282]. This is often done routinely although evidence from clinical research is scarce and often conflicting [283-288]. Although the common notion is that the side effects of corticosteroid treatment are few [282] one has to take into account the substantial existing evidence of impaired wound healing and increased risk of infection [289, 290].

4. Effect of L-NAME on flap blood flow and survival. Paper III

L-NAME, a non-selective inhibitor of NOS, was given as a 100 mg/kg bolus dose followed by a 10 mg/kg/h continuous intravenous infusion, sustained for 60 min in a dorsal, caudally based random skin flap model. The treatment increased blood pressure by approximately 60%, which was expected as L-NAME is known to have a systemic, vasoconstrictive effect [291, 292].

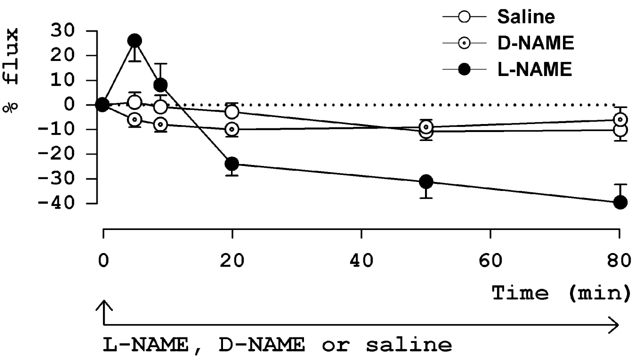

Fig 13. Decreased flap blood flow during L-NAME treatment.

Furthermore, evidence that the treatment also reached the periphery, the skin, was obtained. First, an inhibition of constitutive Ca^{2+} dependent NOS activity in intact skin was registered using citrulline assay. Second, a 25% reduction of intact skin blood flow was seen through laser Doppler measurement.

In the proximal part of the flaps, L-NAME as compared to D-NAME and saline treatments caused a significant inhibition of constitutive Ca^{2+} dependent NOS activity and a significant decrease in blood flow as measured by laser Doppler **(fig 13)**. Finally, the treatment resulted in a significant decrease in flap survival as compared to controls. This decreased flap survival was most probably a result of the inhibition of eNOS activity leading to reduced blood flow and increased aggregation and activation of platelets and leukocytes [111-113].

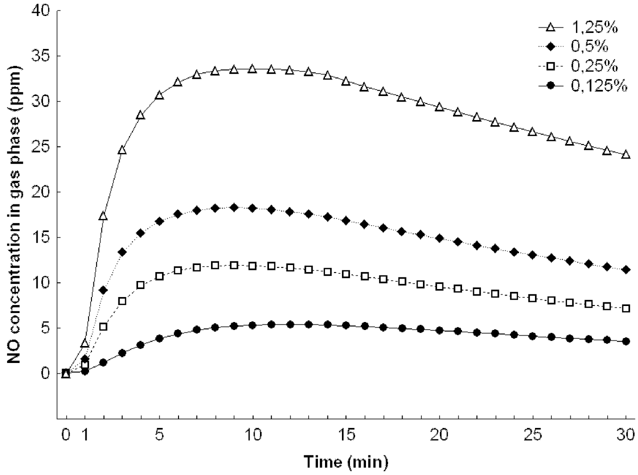

Fig 14. NO-generation from NO_2^- cream acidified with vitamin C.

5. Generation of NO from acidified NO_2^-. Paper IV

The formation of NO from a cream containing increasing concentrations of NO_2^- and vitamin C was demonstrated in vitro using chemiluminescence. Non-enzymatic formation of NO has previously been shown from NO_2^- upon acidification in a multi-step reaction with HNO_2 as an intermediate [132, 133]. If NO_2^- is acidified by vitamin C, as in **Paper IV**, HNO_2 reacts with vitamin C (ascorbic acid, Asc) to form NO, dehydroascorbate (DHAsc) and water [293, 294] **(formulas 4 and 5)**.

$$NO_2^- + H^+ \leftrightarrow HNO_2 \quad (4)$$
$$2HNO_2 + Asc \rightarrow 2NO + DHAsc + 2H_2O \quad (5)$$

The formation of NO was concentration dependent with increasing concentrations of NO_2^- and vitamin C **(fig 14)**.

6. Application of acidified NO_2^- increases flap blood flow. Paper IV

The cream containing acidified NO_2^-, which was shown to generate NO in vitro as described above, was applied to the surface of a modified, ventral, epigastric island skin flap model in the rat for 30 min. Using laser Doppler perfusion imaging technique, superficial capillary blood flow was measured before and after the 30 min treatment. The cream was shown to cause a concentration dependent increase in superficial flap blood flow of up to 120% as seen with the highest (2.5%) concentration. As NO is known to diffuse freely through tissues [225] it most probably penetrated the superficial layers of the flap skin reaching precapillary sphincters and arterioles in the dermis and subcutis thus causing smooth muscle relaxation, vasodilation and increased blood flow.

Besides the measurement of superficial, capillary blood flow, supplying blood flow in the epigastric artery to the flap was measured continuously during the 30 min treatment period using transit-time ultrasound technique (**fig 15**). The treatment caused a concentration dependent increase in supplying blood flow, likely due to decreased resistance in the flap as a result of the above described dilation of smaller blood vessels. During treatment with the three lower NO_2^- concentrations of the NO generating cream, 0.125%, 0.25% and 0.5%, the increase in supplying blood flow was concentration dependent. Treatment with the two highest concentrations, 1.25% and 2.5%, both resulted in an increase of the same magnitude as the 0.5% concentration. These three concentrations thus all caused an approximately 120% increase in blood flow. The two highest concentrations did not increase the supplying blood flow further. The reason for was probably that they also caused a lowered systemic blood pressure resulting in a decreased flap perfusion pressure. The decrease in blood pressure indicates that the NO generated by the cream also had a systemic, vasodilating effect. This vasodilation was probably not elicited directly by NO, as its half-life is relatively short. Instead it could be mediated through the formation of different endogenous NO donors such as SNO-albumin, SNO-Hb, NO-Hb and NO_2^- in the flap [295]. These substances have all been found to be capable of binding NO for possible later release [140-147].

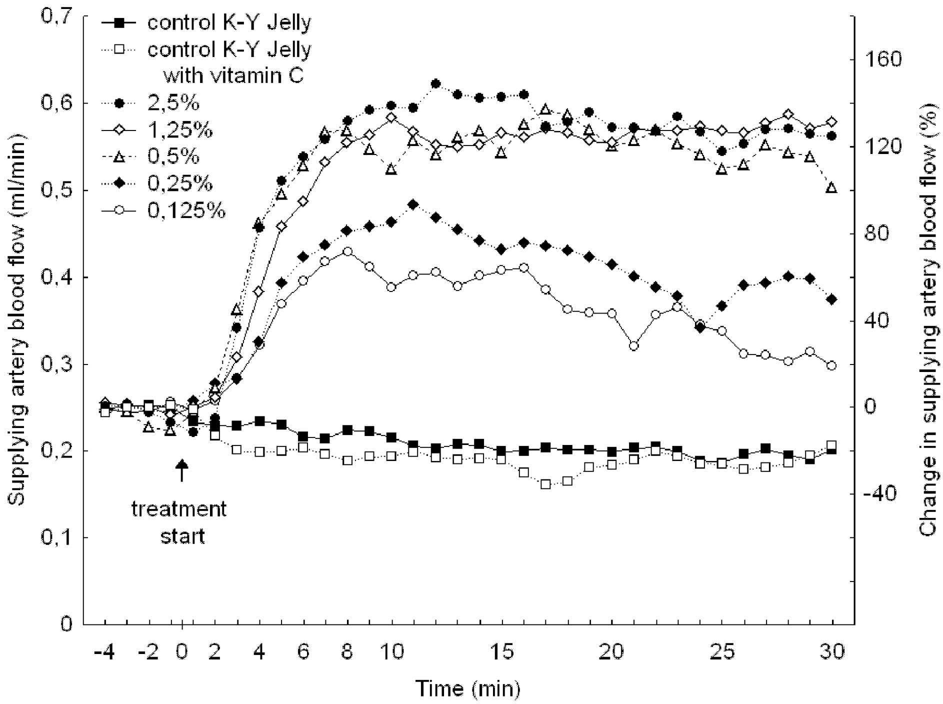

Fig 15. Increased supplying blood flow during topical treatment with NO from acidified NO_2^-.

7. Generation of NO_2 from acidified NO_2^-. Paper IV

In addition to forming NO, the cream containing acidified NO_2^- also produced NO_2. The formation of NO_2 was, just as the formation of NO, dose-dependent with regard to the concentrations of NO_2^- and vitamin C. NO_2 formation is known to occur when NO reacts with oxygen as follows **(formula 6)** [296].

$$2\,NO + O_2 \rightarrow 2\,NO_2 \qquad (6)$$

As seen in the formula, the NO_2 formation depends on the square of the NO concentration, which must be the reason why NO_2 increased so much during the highest NO_2^- concentrations of the cream.

NO_2 is a known toxicant that displays both acute and chronic negative effects on the respiratory tract, which is why exposure limits have been set in many countries [296, 297]. The workplace environmental 8-hour time-weighted limit is 2 ppm in the European Community (International Occupational Safety and Health Information Centre). When using the 1.25% concentration of the cream NO_2 values in the air above the cream exceeded 2 ppm. However, with the three lower concentrations 0.125%, 0.25% and 0.5% released maximally 0.3, 0.7 and 1.2 ppm respectively. Future studies evaluating the final room air concentrations have to be performed in order to, if possible, find a treatment concentration which can be used clinically without being hazardous to the patient and hospital staff.

GENERAL DISCUSSION

Tissue damage caused by ischemia or ischemia and reperfusion injury is a problem in many clinical settings [179-183]. In reconstructive surgery, free flaps are subjected to ischemia from the time the supplying vessels are cut during flap harvest, until blood flow has been re-established through vascular anastomoses at the site of reconstruction. At times post-operative, secondary ischemia is seen in free flaps and is then mostly caused by thrombosis in the flap's artery or vein [64, 65]. The situation can be solved by re-operation with removal of the thrombosis and resuturing of the anastomoses. This will then lead to a second reperfusion period.

The ischemia seen in pedicled flaps is often of a more relative kind, increasing towards the distal end of the flap [185-187].

Ischemia or ischemia and reperfusion occasionally result in partial or total flap failure and necrosis. This is a clinical problem leading to discomfort for the patient, re-operation, increased hospital stay and at worst, the patient's death.

The molecular and cellular mechanisms underlying flap tissue damage due to ischemia and reperfusion are not fully understood. It is known however that endothelial function is altered with a decreased production of vasodilators and increased production of inflammatory mediators [298, 299]. In this thesis the role of NO in experimental skin flaps was explored.

1. Constitutive NOS promoting blood flow

Endothelial cells and neurons are capable of synthesizing NO and do so by using the enzymes eNOS and nNOS respectively. Both isoforms are constitutive, synthesizing NO continuously in small amount. Furthermore, they both require Ca^{2+} for their function and are thus said to be Ca^{2+} dependent [93].

In **Papers I-III** constitutive Ca^{2+} dependent NOS was found in intact rat skin and in **Paper I**, this NOS activity gradually decreased in dorsal flaps with increasing time after surgery, reaching very low levels. The decrease was most rapid in the distal, most ischemic part of the flaps. As eNOS probably is the main form of NOS in flap tissue, the decrease in Ca^{2+} dependent NOS activity was likely caused by damage to the endothelium. This assumption was supported through morphologic studies using TEM where a progressive destruction of the endothelium was seen mainly in the distal part of the flaps.

The effects of NO on smooth muscle relaxation [80] and vasodilation [81] were shown long before the discovery of NO as a biological mediator [85-87]. Subsequently it was realised that NO is of major importance in the regulation of blood pressure and blood flow as NO, continuously released from the endothelium, creates a vasodilator tone in arteries [300, 301]. Inhibitors of NO synthesis have previously been shown to affect this fundamental role of NO thus causing vasoconstriction with an increase in systemic blood pressure and a decreased tissue blood flow as a result [300-303].

To investigate the role of constitutive NOS on flap blood flow and survival, the non-selective NOS inhibitor L-NAME was used in **Paper III**. Intravenously administered L-NAME was here seen to inhibit constitutive NOS activity and cause a marked reduction of blood flow in intact skin and in dorsal flaps. This reduction in blood flow was probably mainly due to inhibition of eNOS and endothelial NO release. NO from eNOS normally accounts for basal vasodilator tonus and its inhibition is known to cause vasoconstriction [302]. Besides being a vasodilator, NO from the endothelium is known to inhibit the adhesion, aggregation and activation of platelets [109, 110] and leukocytes [111, 112]. In **Paper I,** decreasing constitutive NOS activity was paralleled by an accumulation and aggregation of neutrophils and platelets in dorsal flaps. The decrease in flap blood flow seen in **Paper III**, both in untreated flaps and more so in L-NAME treated flaps, could therefore be caused not only by vasoconstriction, but also by an interaction between endothelium, leukocytes and platelets. The accumulation of neutrophils and platelets is known to impede blood flow by decreasing blood fluidity (increased viscosity), by the formation of cellular conglomerates and by the release of vasoconstrictive substances (e.g. LTB_4 and TXA_2) [191, 200, 214].

In **Paper I** studies using TEM showed severe stasis in the distal part of the flaps and this was assumed to result in a nonexistent blood flow. Interestingly, this was later confirmed in **Paper III** where laser Doppler measurements showed a minute flow in the distal part of the flaps. These findings clearly point toward the occurrence of the so-called "no reflow" phenomenon described by other authors [66, 215].

2. iNOS: Tissue destructive?

The inducible form of NOS (iNOS) was first demonstrated in macrophages, which through iNOS produce NO at high concentrations during the killing of pathogens [119, 123]. The enzyme is Ca^{2+} independent and was subsequently found in other types of leukocytes such as neutrophils and monocytes.

In **Paper I** an increase in inducible Ca^{2+} independent NOS activity was seen in dorsal flaps and in **Paper II** dexamethasone inhibited this induction and increased flap survival. The results show that dexamethasone is positive to flap survival and that induction of iNOS could be negative.

The role of NO during ischemia and reperfusion is not fully understood. However, it seems clear that high concentrations of NO synthesized by iNOS, mainly from leukocytes, can be tissue damaging [217, 225]. This was in part supported in **Paper I** where iNOS induction and increase in the number of neutrophils and macrophages was seen parallel to evidence of increasing tissue damage. In addition other cells such as endothelial cells and fibroblasts could be the source of iNOS [124, 125].

Ischemia and reperfusion injury, through the formation of different cytokines, constitutes a powerful stimulus for iNOS. The damaging effects of high NO concentration occur both directly and via the formation of tissue damaging free radicals. Direct damaging effects of NO have been seen on DNA through strand breaks and inhibition of DNA-synthesis [225, 304, 305]. Furthermore, NO interacts with mitochondrial cytochrome c oxidase, an enzyme of the mitochondrial respiration chain. Thereby NO might inhibit oxygen transport and block cellular respiration [223, 306-308]. The indirect damaging effects of NO occur via different NO derived free radicals. The probably best known is peroxynitrite ($ONOO^-$), which is formed through the reaction between NO and superoxide (O_2^-). O_2^-, a free radical in itself, is produced in abundance upon reperfusion mainly by NADPH oxidase. Other examples of NO derived free radicals are peroxynitrous acid (HOONO), hydroxyl radical (OH) and nitrous anhydride (N_2O_3). These free radicals are able to cause DNA damage, induce lipid peroxidation and limit enzyme functions [225, 309, 310]. Also, during ischemia and reperfusion NOS has been seen to change its function and start synthesizing the tissue-damaging free radical O_2^- instead of NO, an event known as uncoupling of NOS [96]. O_2^- generation from NOS occurs both when the NOS cofactor BH_4 is converted to BH_2 (as described earlier) and also in the lack of the NOS substrate L-Arg [311]. It is possible that there is a lack of L-Arg in ischemic flaps and that this might lead to O_2^- generation by iNOS. Whether such a decrease in L-Arg can be compensated for by L-Arg administration remains unclear to us. We have, in contradiction to others [234, 235], not been able to prove that L-Arg administration is positive to flap survival (unpublished results).

It is therefore possible that the increased survival of flaps in **Paper II** could be achieved through the inhibition of iNOS by dexamethasone thus preventing the above described negative effects of NO. However, other anti-inflammatory and positive effects of dexamethasone such as inhibition of leukocytes accumulation and activation have to be taken into account [277].

Increasing evidence indicates that induced NO synthesis by iNOS under hypoxia and ischemia might not be tissue destructive, but instead tissue protective. NO has thus been found to act as a free radical scavenger neutralising O_2^-, $ONOO^-$, OH and iron related radicals. NO is also capable of preventing the stimulation of genes that encode the synthesis of pro-inflammatory cytokines, adhesion molecules and cyclooxygenase. Furthermore, NO has been seen to prevent lipid and lipoprotein peroxidation and inhibit NADPH oxidase (producer of O_2^-) [217, 309, 310, 312, 313]. Put into this light, the conclusion could be that the inhibition of iNOS by dexamethasone in **Paper II** only was an epiphenomenon or possibly negative to the flaps. Also, induction of iNOS in flaps as observed in **Paper I** might be an attempt to salvage the ischemic tissue.

It thus seems that NO has opposing and contradictory effects making it hard both to understand the role of NO during ischemia and reperfusion and also difficult to design treatment drugs. An interesting approach would be to inhibit the negative effects of iNOS without inhibiting its positive effects. The answers will probably be found within the biochemistry of NO and in the increased understanding of factors in the tissue itself. The way NOS produces varying effects is by synthesizing NO at different rates and in different locations. In the tissue, factors such as oxygen level, redox state, acidity, the concentrations of NO scavengers (e.g. hemoglobin), antioxidants (e.g. SOD and glutathione) and other reactive molecules (e.g. O_2^-) all influence the final effect of NO [217, 220, 221, 225, 309, 314].

An example of this complex interplay is the formation of $ONOO^-$ from NO and O_2^-. For this to occur, it is believed that NO and O_2^- have to be produced at similar concentrations in a similar location [309]. Both NO and O_2^- are efficient scavengers of $ONOO^-$, which is why a great surplus of either NO and O_2^- will lead to the decomposition of formed $ONOO^-$. The macrophage is an efficient producer of $ONOO^-$ as it forms NO and O_2^- at similar concentrations simultaneously. In contrast, neutrophils produce O_2^- at much higher concentrations than they do NO, and therefore do not produce $ONOO^-$ efficiently [309]. O_2^- from neutrophils is however tissue damaging in itself [315]. Furthermore, as NO is capable of reacting with O_2^-, the bioactivity and positive effects of NO seem to depend on O_2^- concentrations and also on concentrations of SOD, the most potent scavenger of O_2^- [316].

Additional insight into the role of NO during ischemia and reperfusion is probably found in the regulation of different pro-inflammatory genes. The transcription factor NF-kappa B, which is stimulated during oxidative stress, is an important activator of genes expressing pro-inflammatory agents such as cytokines, adhesion molecules, cyclooxygenase and also iNOS. NO is capable of inhibiting NF-kappa B thus decreasing inflammation and acting as a negative feed-back for iNOS. Also, NO has been shown to stimulate NF-kappa B, thereby causing an increased inflammatory response [223, 313].

3. Treatment with NO

Treatment with NO as a remedy started, through the use of amyl nitrite and nitroglycerine against angina pectoris [154, 155], long before the discovery of NO as a biological mediator. During recent decades the number of drugs related to NO has increased greatly and are now used in the treatment of conditions such as respiratory distress syndrome (RDS), pulmonary hypertension, systemic hypertension, arteriosclerosis, erectile dysfunction and inflammatory conditions [153, 164, 317, 318]. Treatment with gaseous NO to a tissue is effective as long as it does not come in contact with the scavenging effect of Hb and is attractive since it can be expected to have fewer effects on the systemic circulation as compared to for example infusion of known NO donors. The lung is thus accessible for this type of treatment [153]. Another organ which is easily accessible is the skin, which can be treated through topical application of NO releasing drugs. In this context topical nitroglycerine had been demonstrated to increase the survival of experimental skin flaps [319]. We were however unable to reproduce this beneficial effect but noted a marked reduction in systemic blood pressure (unpublished results). Non-enzymatic generation of gaseous NO from acidified NO_2^- was first discovered in the stomach [132, 133]. Subsequently acidified NO_2^- has been used topically on the skin to treat cutaneous leishmaniasis and Raynaud's syndrome. [268, 269] Using this information a NO releasing cream containing acidified NO_2^- was tested on an island flap model in **Paper III**. At the lower NO_2^- concentration, the cream was found to efficiently increase flap blood flow without causing any systemic effects. The highest concentrations did, however, reduce blood pressure slightly. As expected the noxious gas NO_2 was also generated probably making further modifications to the cream necessary before the cream can reach clinical practice. Topical application of a treatment drug to skin flaps is an interesting concept and topical treatment with an NO releasing compound could prove valuable to substitute for the decrease in constitutive NOS activity found in **Paper I.**

4. NO and flap survival

In the current thesis the role of NO in skin flaps has been studied. Inhibition of constitutive NOS, probably eNOS, decreased flap survival and inhibition of iNOS was seen parallel to increased flap survival. The results suggest that NO released in small amounts from the endothelium is tissue protective and that large amounts of NO from iNOS released by leukocytes is tissue destructive. Protective effects of low amounts of NO from eNOS include vasodilation and inhibition of platelet and leukocyte activity. Large amounts of NO from iNOS causes tissue damage both directly by itself and also indirectly through the formation of free radicals.

Modulation of the NO system in ischemic flaps has during the course of this thesis work been studied by others. The effects of NO donors [236-238], NOS inhibitors [228-233], iNOS gene knock-out [239, 240], L-Arginine

[234, 235] and phosphodiesterase inhibition [320, 321] have all been studied and many of the results are conflicting. However, explanations will probably be found in the future. Tissues, including flap tissues, differ and slight variations in oxygen level, pH, redox state and NO scavenger concentrations all affect the role of NO. Furthermore, the effects of NO depend on its release profile and its local concentration [217, 225, 309].

Future studies will increase our knowledge on the role of NO during ischemia and reperfusion and it is likely that there are NO related treatment methods against flap failure and necrosis around the corner.

CONCLUSIONS

- endogenous NO formation increases flap blood flow and promotes flap survival in experimental skin flaps in the rat
- ischemia in experimental skin flaps in the rat leads to a reduction of constitutive NOS and causes an induction of iNOS
- dexamethasone attenuates the induction of iNOS in experimental skin flaps in the rat and promotes flap survival
- the endogenous NO, which promotes flap survival may be formed both by eNOS and iNOS, but the overall effects of iNOS in severely affected flap areas seem detrimental
- exogenous NO increases the blood flow in experimental skin flaps in the rat
- local administration of NO to experimental skin flaps in the rat can be achieved through the use of a topically applied NO generating cream, which in the future may be used to promote blood flow in surgical flaps in the clinical setting
- flap ischemia causes endothelial damage, accumulation of platelets and neutrophil granulocytes and congestion of blood vessels
- future research could be aimed at investigating to what degree these changes are a result or a cause of changes in flap biology

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SUMMARY IN SWEDISH

Kirurgiska lambåer används inom plastikkirurgin för att rekonstruera vävnadsdefekter uppkomna efter exempelvis trauma eller tumöroperation. Härvid flyttas vävnad såsom hud, fett, muskel och ben kortare eller längre distanser (stjälkade respektive fria lambåer). Ibland utsätts lambåer för allvarlig ischemi (blod- och syrebrist) vilket kan leda till vävnadsskada och lambådöd.

Gasen kväveoxid (NO) har stor betydelse i kroppen då den bland annat deltar i reglering av blodtryck, regionalt blodflöde, nerv-signalering och immunsvar. NO bildas av enzymet NO syntas (NOS) som föreligger i konstitutiva och inducerbara former. Konstitutivt NOS i endotelet (det cellager som bekläder insidan av alla kärl) bildar kontinuerligt NO i små mängder. NO sprider sig härefter till den glatta muskulaturen i kärlväggen som fås att slappna av. Blodkärlen vidgas och på så sätt har NO kontinuerligt en blodtryckssänkande effekt och kan dessutom öka blodflödet till olika vävnader. NO från endotelet förhindrar dessutom vidhäftning av vita blodkroppar och blodplättar (trombocyter) till kärlväggens insida. Inducerbart NOS (iNOS) i vita blodkroppar är normalt sett inaktivt men stimuleras av bakterier och inflammatoriska mediatorer till att bilda höga koncentrationer av NO. Höga NO-koncentrationer kan vara cellskadande dels i sig och dels via bildning av fria radikaler. De vita blodkropparna utnyttjar detta för att bekämpa exempelvis bakterier och parasiter. Dessutom kan de höga koncentrationerna av NO med bildning av fria radikaler orsaka vävnadsskada till exempel i samband med ischemi och reperfusion (återupprättat blodflöde).

Skada på grund av ischemi och reperfusion ses kliniskt i olika vävnader såsom hjärnan (stroke), hjärtat (hjärtinfarkt) och lambåer (lambådöd). Orsakerna till att ischemi och reperfusion leder till vävnadsskada är inte klarlagda och det vore av stort kliniskt intresse att här både få en ökad insikt samt att finna lämpliga behandlingsmetoder.

I denna avhandling användes experimentella lambåer på råtta, med reproducerbar och standardiserad blod-försörjning, ischemi och lambådöd. I modellerna studerades vilken roll NO har för lambåcirkulation och lambåöverlevnad. NOS-aktivitet, lambåmorfologi, lambåblodflöde och lambåöverlevnad mättes efter hämning av NOS och administrering av NO.

I intakt hud noterades (via citrullinassayteknik) konstitutiv NOS-aktivitet och denna NOS-aktivitet sågs sedan gradvis minska i ischemisk lambåvävnad med ökat antal timmar efter operation. Parallellt noterades (via elektronmikroskopi) tecken till endotelcellsskada. Vid hämning av konstitutivt NOS med hjälp av NOS-hämmaren L-NAME minskade blodflödet och överlevnaden hos lambåerna.

Ingen iNOS-aktivitet uppmättes i intakt hud. Däremot sågs en induktion av iNOS i ischemiska lambåer. Samtidigt sågs ansamling av vita blodkroppar och blodplättar. Behandling med dexametason hämmade iNOS och en ökad lambåöverlevnad.

Förutom att NO bildas enzymatiskt via NOS, kan NO även bildas icke-enzymatiskt från nitrit (NO_2^-) i sur miljö. En kräm med nitrit och c-vitamin (surt, pH 5) bereddes och sågs (via mätning med kemiluminesens) bilda NO och även NO_2 (som bildas då NO reagerar med luftens syre). Krämen applicerades på en ö-lambåmodell varvid ett ökat blodflöde sågs i lambåytan och försörjande blodkärl (med hjälp av laser-Doppler respektive ultraljudsteknik).

Sammanfattningsvis visar resultaten att konstitutivt NO, troligen framför allt från eNOS i endotelet, är viktigt för lambåöverlevnad genom att vidmakthålla lambåblodflöde och troligen även genom att förhindra ansamling och aktivering av vita blodkroppar och blodplättar. Dessutom indikerar resultaten att iNOS, som har förmågan att bilda toxiskt höga koncentrationer av NO, är negativt för vävnaden. Det är dock känt att NO från iNOS även kan vara vävnadsskyddande och den slutgiltiga effekten av NO är komplex.

Hämning av de skadliga effekterna av iNOS och behandling med NO för att kompensera minskad konstitutiv NOS-aktivitet kan visa sig gynnsamt för lambåer och kan komma att utgöra behandlingsmetoder mot lambåischemi och lambådöd hos patienter. Administrering av NO lokalt, till exempel via en kräm, är ett attraktivt behandlingsalternativ.

I don't want to achieve immortality through my work. I want to achieve it through not dying.

Woody Allen