Vascular effects of nitric oxide the relation to migraine

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Vascular Effects of Nitric Oxide
The Relation to Migraine

De vasculaire effecten van stikstof oxide
de relatie met migraine

Proefschrift

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Ter nagedachtenis aan mijn vader
Voor Saskia, Kimberley en Felix

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Chapter 1

General introduction

1.1 Pathophysiology of migraine

Migraine may manifest itself in many ways and the syndrome is afflicting approximately ten percent of the worlds population. The peak incidence of the syndrome occurs in adolescents and young adults, and the prevalence is about three times as high among females than males (Staffa *et al.*, 1994). The diversity of symptoms and the potential confusion with other types of headaches, such as tension-type headache and cluster headache, has lead to the formulation of diagnostic criteria by the International Headache Society (Olesen *et al.*, 1988). These criteria discern between two main types of migraine. The first, migraine without aura, was previously known as common migraine and is characterized by recurrent attacks of intense headache lasting 4 to 72 hours. The headache is commonly unilateral, pulsatile and throbbing in nature. The pain is often accompanied by anorexia, nausea and vomiting, as well as aversion to noise and light (phonophobia and photophobia). In the second type, previously known as classical migraine, headache is preceded by neurological symptoms called aura symptoms. The classic aura includes visual disturbances, like scintillating scotoma and fortification spectra, that drift across the visual field. In addition, somatosensory symptoms may occur as a feeling of numbness (pins and needles) slowly ascending from the fingertips to the shoulder (Spierings, 1988; Blau, 1992). The duration of the aura phase is often limited to 60 minutes, but may outlast into the headache phase.

The clinical forms of migraine attacks suggest that migraine represents in fact two different types of disease. This view is supported by experimental data, demonstrating that cerebral blood flow is usually reduced in migraine with aura but not in migraine without aura (Olesen *et al.*, 1981; Lauritzen, 1994). The difference between the two forms may be attributed to individual variations in reactivity towards the same initial trigger.

Despite detailed descriptions of the events occurring during the actual attack, little is known about the stimulus initiating the sequence of events. A number of prodromal

symptoms have been related to migraine, which include episodes of emotional and physical stress, sleep deprivation, enheightened sensory perception and craving for specific foods (Blau, 1992). The wide variety of these prodromes seems to preclude a common generator of migraine, although some of these stimuli could affect specific brain areas.

The aura

The occurrence of cerebral hypoperfusion during the migraine aura has been documented but the underlying mechanism remains a matter of ongoing debate. At the very beginning of migraine attacks regional cerebral blood flow decreases in the posterior part of the brain. Subsequently, the low flow region spreads into the parietal and temporal lobes at a rate of 2-3 mm min^{-1} (Olesen *et al.*, 1981; for review see, Lauritzen, 1994). It has been argued that this spreading oligemia can not account for the focal symptomes of migraine aura. The spreading oligemia continues after the symptoms of aura have vanished and blood flow reduction is too moderate to cause neuronal dysfunction. Furthermore, the propagation of visual disturbances occurs at a similar rate as cortical spreading depression, a process of cortical neuronal depolarization which can be observed in animals following noxious stimuli, suggesting a neuronal rather than a vascular origin of migraine aura (for review see Lauritzen, 1994). However, re-evaluation of blood flow data taking into account the methodological implications of Compton scatter, that is the contribution of radiation from surrounding tissues to local blood flow analysis, suggested that cerebral blood flow can reach ischaemic levels and thus cause neuronal changes (Olsen & Lassen, 1989). Moreover, cortical spreading depression is not easily observed in humans (Piper *et al.*, 1991).

The headache

The pulsatile and throbbing nature of the headache suggests that a dilatation of large cranial and extracranial vessels is involved in the headache phase of migraine. Indeed, dilatation and enhanced vascular pulsations of the temporal and occipital branches of the external carotid artery, on the side of the headache, were described already in 1938 by Graham and Wolff. Headache relief, obtained with ergotamine, was associated with a reduction in vascular pulsatility. In contrast, administration of histamine caused headache and increased the amplitude of pulsations in the temporal artery (Graham & Wolff, 1938). Reviewing their experimental data, Drummond and

Lance (1988) concluded that an increase in vascular pulse amplitude in the frontotemporal region is observed in approximately one-third of the patients suffering unilateral migraine. More recently, transcranial Duplex-Doppler methods have been applied to monitor changes in blood flow velocity during migraine attacks. In 1990, Iversen reported that the lumen of the superficial temporal artery was wider on the side of headache than on the other side. Likewise, Thie et al. (1990) observed a reduction of blood flow velocity with increased pulse wave amplitudes, in a number of cranial arteries during attacks of migraine without aura. However, evidence of middle cerebral artery dilatation, on the side of migraine headache is still conflicting. A reduction of blood flow velocity without concomitant change in total cerebral blood flow, indicating vasodilatation in this artery, has been observed in a small group of patients (Friberg *et al.*, 1991). In contrast, no changes in middle cerebral artery blood flow velocity were found during and between attacks in patients without aura (Zwetsloot *et al.*, 1993). Despite these equivocal changes, an increase in blood flow velocity in large cranial arteries has been observed after acute treatment with the antimigraine drug sumatriptan (Friberg *et al.*, 1991; Caekebeke *et al.*, 1992).

In order to explain the changes in blood flow and the facial pallor observed in some migraine patients, Heyck hypothesized that enhanced shunting of blood through arteriovenous anastomoses may lead to passive increases in blood flow through large (extra)cranial arteries. At the same time, increased arterial pulsations will propagate, unattenuated, to precapillary vessels as well as the venous side of the circulation (Heyck *et al.*, 1969). This postulate is largely based on the observation that the difference in oxygen content of arterial and venous blood obtained from the ipsilateral side of the head was reduced. Moreover, restoration of the difference in oxygen saturation was observed following either spontaneous or ergotamine-induced headache relief. In line with this view, antimigraine drugs like ergotamine, dihydroergotamine and sumatriptan cause a constriction of arteriovenous anastomoses and large arteries in the porcine carotid circulation (Den Boer *et al.*, 1991, 1993). Heyck's hypothesis is supported by recent observations indicating that human peripheral arteriovenous anastomoses are indeed dilated during migraine attacks and a therapeutic dose of sumatriptan can reduce sodium nitroprusside-induced elavated anastomotic blood flow in the human finger vasculature (Van Es *et al.*, 1995).

Although evidence indicating a direct link between the vascular changes and the incidence of pain is absent, local vasodilatation together with increased vascular pulsations may cause headache via a depolarization of perivascular sensory nerves.

An alternative theory on the pathogenesis of migraine has been put forward by Moskowitz (1992) stating that an unknown trigger causes activation of trigeminal nerve endings on the cranial vasculature leading to antidromic release of vasoactive neuropeptides. Such neuropeptides, like substance P and calcitonin gene-related peptide (CGRP), present in adventitial nerves surrounding cranial arteries (Uddman *et al.* 1986; Suzuki *et al.*, 1989), may induce a temporary aseptic inflammatory process, finally resulting in pain. These peptides are potent vasodilators (Brain *et al.*, 1985; this thesis, chapter 7) and may, therefore, give rise to a concomitant dilatation of large cranial arteries and arteriovenous anastomoses. The possible pathophysiological changes during migraine attacks are summarized in figure 1.1.

Arteriovenous anastomoses

Arteriovenous anastomoses are precapillary blood vessel elements connecting arteries and veins. The localization of these communications enables the blood flow to bypass the capillary bed. These structures have been localized in various tissues including the human and porcine skin, tongue and dura mater. In contrast, no arteriovenous anastomoses have been detected in the brain (Rowbothan & Little, 1965; Kerber & Newton 1973; Den Boer *et al.*, 1992a). Within the skin, arteriovenous anastomoses contribute to the exchange of body heat, whereas in other tissues they may serve to regulate local blood flow. In the open state, the lumen of arteriovenous anastomoses may exceed the diameter of the capillaries and large amounts of blood can be directed either from deep layers to the surface or from the arterial towards the venous side of the circulation.

Depending on the type of tissue, arteriovenous anastomoses are innervated by catecholaminergic nerves, containing noradrenaline, dopamine and/or neuropeptide Y, and cholinergic nerves, containing acetylcholine and vasoactive intestinal peptide (for reviews see Hales & Molyneux, 1988; Den Boer, 1993). In addition, histochemical studies in the dog and sheep tongue have shown the presence of peptidergic nerve fibres, containing CGRP and substance P, around the walls of arteriovenous anastomoses (Molyneux & Haller, 1987; Hino *et al.*, 1993). Preliminary observations indicate that CGRP may also contribute to the regulation of blood flow through arteriovenous anastomoses in the sheep limb (Mogg *et al.*, 1992). Finally, arteriovenous anastomoses may also be under hormonal constrictor influences, since 5-HT and related agonists constrict arteriovenous anastomoses (Saxena & Verdouw, 1982).

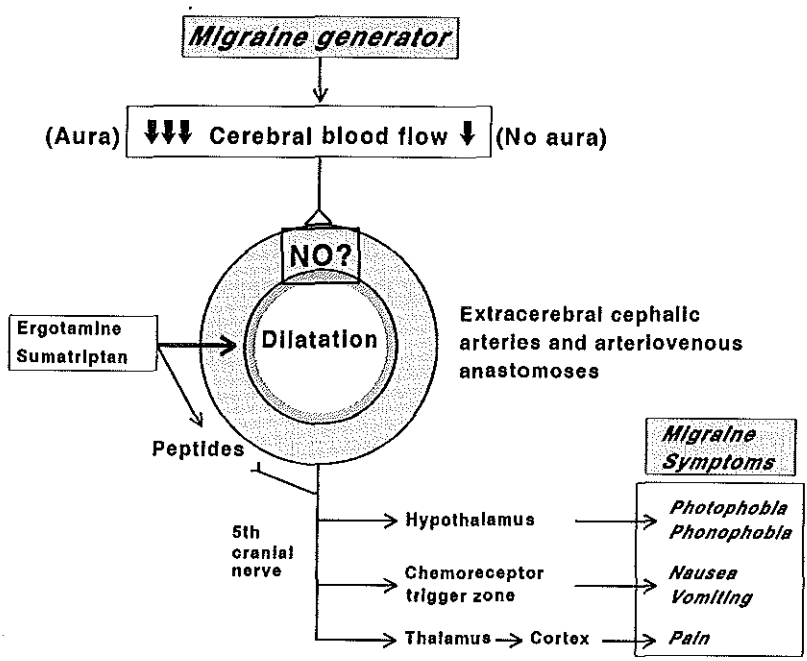

Figure 1.1 Possible changes in migraine. Migraine attacks are initiated by an unknown generator. Cerebral blood flow is reduced during the pre-headache phase, whereas dilatation of extracerebral cephalic arteries and arteriovenous anastomoses occurs during headache. Activation of the trigeminal nervous system (5th cranial nerve) and central pathways may evoke typical migraine symptoms and the release of vasoactive neuropeptides (modified from Saxena, 1994).

1.2 Local regulation of vascular tone; endothelium-derived factors

Introduction

Because of its key localization and physiological functions, the vascular endothelium has been recognized as a regulatory organ rather than a simple sheet of cells aligning the vascular smooth muscle. Vascular endothelium has a variety of physiological functions which include, among others, a selective barrier function, regulation of inflammatory responses and cell proliferation. In addition, the endothelium maintains a nonadhesive luminal surface and, regulates platelet

aggregation and leucocyte adhesion (Radomski *et al.*, 1987; Kubes *et al.*, 1991). Furthermore, endothelial cells contribute to cardiovascular homeostasis via the release of a number of vasoactive substances with opposite actions to regulate the underlying smooth muscle tone and hence local blood flow. Similar to other major organs, endothelial cells receive input from their environment, that is, via circulating or locally produced vasoactive substances and mediators derived from blood cells like platelets and leucocytes. Moreover, the vascular endothelium is stimulated by mechanical forces like shear stress and pressure, provoked by the circulating fluid stream.

Interest in the regulatory functions of endothelial cells was increased substantially by the observation of Furchgott and Zawadzki (1980) that the presence of vascular endothelium is a prerequisite for the vasorelaxant action of acetylcholine. Removal of the endothelial layer from the isolated rabbit aorta prevented acetylcholine-induced relaxations in this vessel and, as relaxations did not depend on the release of cyclo-oxygenase derivatives, it was proposed that other diffusible factor(s) were released by the endothelium. This factor was later termed endothelium-derived relaxing factor (EDRF) and its chemical nature has been examined in detail. The preponderance of evidence suggests that EDRF is chemically related to the nitric oxide radical. In addition to EDRF, the existence of other endothelium-derived relaxing factors have been recognized which include the endothelium-derived hyperpolarizing factor (Garland *et al.*, 1995) as well as the metabolite of arachidonic acid, prostacyclin (Vane *et al.*, 1990). In parallel with the discovery of endothelium-derived relaxing factors a host of contracting factors (EDCFs), have been postulated in the last decade. Apart from a number of unidentified constrictor substances, two major categories can be recognized. The first group includes products of the cyclo-oxygenase pathway such as thromboxane, prostaglandin H_2 and superoxide anions (Vane *et al.*, 1990; Lüscher *et al.*, 1992). The second group consists of the family of potent vasoconstrictor isopeptides, the endothelins (Yanagisawa *et al.*, 1988).

The simultaneous release of relaxing and contracting factors, stimulated by the same naturally occurring substances, gives rise to a variety of interactions, and their opposite actions seem to imply a delicate balance under physiological conditions. As a consequence, local changes in endothelial function may have considerable implications on local blood vessel tone and blood flow.

The following sections summarize the various endothelium-derived contracting and relaxing factors with special emphasis on the current knowledge of the vascular physiology of nitric oxide.

Endothelium-derived constricting factors

Prostanoids

In a variety of blood vessels, endogenous agonists evoke endothelium-dependent contractions which can be blocked by inhibitors of the enzyme cyclooxygenase. This suggests that the endothelial cell is the source of these mediators. The major vasoconstrictor product of cyclooxygenase is thromboxane A_2. Its formation from endoperoxides is catalyzed by the enzyme thromboxane synthetase (Moncada & Vane, 1979). Since the production of thromboxane A_2 cannot explain all endothelium-dependent contractions, other metabolites of arachidonic acid have been proposed to be involved. For example, in the dog cerebral artery contractions have been attributed to the precursor of thromboxane A_2, prostaglandin H_2 (Toda *et al.*, 1988), whereas in the canine basilar artery superoxide anions produced by the cyclooxygenase-pathway seem to mediate contraction via inactivation of endothelium-derived relaxing factor (Katusic & Vanhoutte, 1989).

Endothelins

This group of vasoactive isopeptides is probably one of the most impressive representatives of the EDCFs. The members of this family, denoted ET-1, ET-2 and ET-3 respectively, are generated from the precursor molecules prepro-endothelin and proendothelin (big endothelin). Conversion of big endothelin to the vasoactive 21-aminoacid peptides is mediated in endothelial cells by the metalloprotease enzyme endothelin-converting enzyme (ECE), which process is blocked by phosphoramidon (Yanagisawa *et al.* 1988; Matsumura *et al.*, 1990). The release of endothelin from the vascular endothelium is provoked by vasopressor substances like angiotensin II and vasopressin, the coagulation product thrombin as well as by brief periods of anoxia and vascular stretch (Figure 1.2; for review see Lüscher *et al.*, 1992; Macarthur *et al.*, 1994).

When injected intravenously, endothelins elicit a biphasic blood pressure response, that is, a brief depressor phase followed by a long-lasting pressor response (DeNucci *et al.*, 1989). The various receptors for endothelin have been cloned (Arai *et al.*, 1990; Sakurai *et al.*, 1990) and according to the currently held receptor classification the pressor response is mediated via stimulation of endothelin ET_A and ET_B receptor subtypes present on vascular smooth muscle cells (Figure 1.2). In contrast, the short-lasting depressor response is mediated via endothelin ET_B receptors on the

vascular endothelium (Davenport & Maguire, 1994). The vasorelaxant action of endothelin has been attributed to a concomitant release of prostacyclin and EDRF which may limit endothelin induced vasoconstriction (DeNucci *et al.*, 1988; Warner *et al.*, 1992; Fozard & Part, 1992). In addition, the production of endothelin is regulated by inhibitory stimuli like prostacyclin, EDRF and ADP, presumably via a cGMP-dependent mechanism (Boulanger & Lüscher, 1990; Saijonmaa *et al.*, 1990).

Under normal conditions, circulating levels of endothelin are very low, which seem to imply that the peptide has a moderate role in the regulation of vascular tone. However, ET-precursor mRNA is expressed constitutively within the vascular wall and, the ECE inhibitor phosporamidon as well as the endothelin ET_A receptor antagonist BQ123 have been shown to increase regional blood flow in the human forearm suggesting that endothelin is released continuously in the vasculature (Haynes & Webb, 1994). Moreover, endothelin is released predominantly towards the abluminal side of the blood vessel and the peptide may be regarded as a local regulatory substance rather than a circulating hormone (Lüscher *et al.*, 1992). The potent vasoconstrictor properties of endothelin have been related to a number of pathological conditions, including migraine (Färkkilä *et al.*, 1992; Gallai *et al.*, 1994).

Angiotensin

Though angiotensin II was initially recognized as a circulating vasoconstrictor peptide mainly derived from the kidneys, endothelial angiotensin converting enzyme (ACE) has been implicated in the conversion of angiotensin I to angiotensin II. Inspite of an ongoing debate on the origin of vascular renin, major components of the renin-angiotensin system, like angiotensinogen, have been identified in cultured endothelial cells and these cells are capable to produce angiotensin II (for review see Dzau, 1989; Von Lutterotti *et al.*, 1994). In this respect, angiotensin II can also be regarded as an endothelium-derived contracting factor. Pharmacological evidence suggests that at least two different angiotensin receptor subtypes (AT_1 and AT_2) exist in the peripheral circulation and the brain (for review see Timmermans *et al.*, 1993). Vascular smooth muscle cells predominantly express angiotensin AT_1 receptors and activation of this receptor subtype commonly produces vasoconstriction (Figure 1.2). In addition to its hypertensive action, angiotensin II can produce endothelium dependent relaxation via yet undefined receptor subtypes.

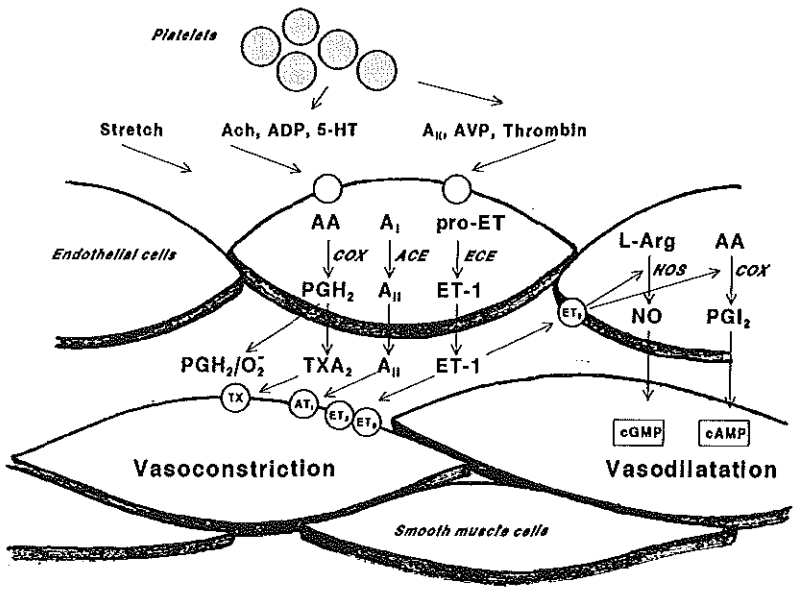

Figure 1.2 Endothelium-derived contracting factors. Stimulation of cyclooxygenase (*COX*) by agonists, partly derived from aggregating platelets, and stretch induces the release of thromboxane A_2 (TXA_2), prostaglandin H_2 (PGH_2) and superoxide anion (O_2^-), which mediate contraction. Similarly, activation of endothelin-converting enzyme (*ECE*) leads to the production of endothelin (ET-1). ET-1 may activate smooth muscle endothelin ET_A and ET_B receptors to mediate contraction. Stimulation of endothelial ET_B receptors initiates the release of endothelium-derived relaxing factors NO and prostacyclin (PGI_2), which cause vasorelaxation. Local conversion of angiotensin I (A_I) into angiotensin II (A_{II}) by angiotensin-converting enzyme (*ACE*) may induce AT_1 receptor mediated smooth muscle contraction. Abbreviations: AA, arachidonic acid; Ach, acetylcholine; AVP, arginine vasopressin; L-Arg, L-arginine; *NOS*, NO-synthase; 5-HT, 5-hydroxytryptamine; pro-ET, pro-endothelin.

Endothelium-derived relaxing factors

Prostacyclin

The potent vasorelaxant and platelet-inhibitory metabolite of arachidonic acid, prostacyclin, is produced by endothelial and vascular smooth muscle cells. Its formation involves conversion of arachidonic acid to endoperoxide prostaglandins G_2

and H_2 which are subsequently converted by prostacyclin synthase to prostacyclin (Vane *et al.*, 1990). The release of prostacyclin from the vascular endothelium is stimulated by several endogenous mediators, like bradykinin, thrombin and endothelin, platelet-derived substances like 5-HT and adenine nucleotides as well as by anoxia and physical forces such as shear stress (Figure 1.3). The endothelial production of prostacyclin is activated by similar stimuli as EDRF/NO production, indicating that these substances may act in concert to reduce vascular tone.

Endothelium-derived hyperpolarizing factor

In arterial tissues, cholinomimetics and other agonists like histamine and substance P, cause vasorelaxation which is often accompanied by an increase in smooth muscle membrane potential. This hyperpolarization is converted into depolarization after removal of the endothelium, thereby suggesting the release of a hyperpolarizing factor from endothelial cells (Komori *et al.*, 1988; Chen & Suzuki, 1989; for review see Garland *et al.*, 1995). The chemical identity of this factor, termed endothelium-derived hyperpolarizing factor (EDHF) and the cellular mechanism mediating endothelium-dependent hyperpolarization are distinct from those of EDRF and cyclooxygenase products. For example, hyperpolarization in response to acetylcholine is largely unaffected by agents, like haemoglobin, methylene blue and inhibitors of NO-synthase, known to inhibit EDRF-mediated relaxant responses (Chen & Suzuki, 1989; Garland & McPherson, 1992). The change in smooth muscle potential evoked by EDHF has been associated with an increase in K^+ conductance (Chen & Suzuki, 1989) through small conductance Ca^{2+}-activated K^+ channels, although the type of channel involved is contentious. Comparison of agonist-evoked hyperpolarization and relaxation in isolated blood vessels of different size suggests that EDHF may have a predominant role in small arterioles. In contrast, hyperpolarization and relaxation in large conduit arteries has been ascribed largely to EDRF (see Garland *et al.*, 1995). The physiological role of smooth muscle hyperpolarization remains to be clarified.

Nitric oxide

Discovery

Following the discovery of EDRF, the labile nature of this factor became evident from cascade bioassay experiments in which a series of endothelium-denuded detector vessels relaxed following superfusion with the perfusate from endothelium-intact donor

blood vessels or cultured endothelial cells. Variation of the distance between donor and detector preparation enabled detection of its biological half-life, ranging from 4 to 6 seconds (Griffith *et al.*, 1984; Rubanyi *et al.*, 1985; Gryglewski *et al.*, 1986; Palmer *et al.*, 1987).

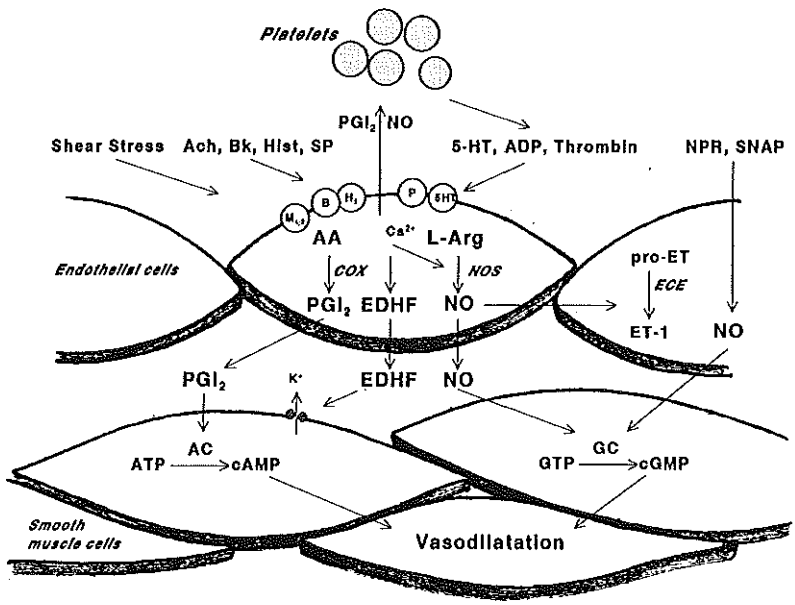

Figure 1.3 Endothelium-derived relaxing factors. Various substances, partly derived from aggregating platelets and, shear stress may, in part via specific receptors, evoke the release of PGI_2 and NO from endothelial cells. In resistance vessels, endothelium-derived hyperpolarizing factor (EDHF) is released. The formation of PGI_2 from arachidonic acid (AA) is mediated by cyclooxygenase *(COX)*. Its release leads to an increase in cAMP content of vascular smooth muscle cells. Calcium-dependent NO-synthase *(NOS)* produces NO from L-arginine (L-arg). NO stimulates smooth muscle guanyl cyclase (GC) to increase cytoplasmatic cGMP content, which action is mimicked by nitrovasodilators (nitroprusside sodium (NPR) and S-nitroso-N-acetylpenicillamine (SNAP)). EDHF may induce vasorelaxation by increasing smooth muscle cell K^+ conductance and subsequently reducing intracellular free Ca^{2+} levels. In addition, both PGI_2 and NO inhibit platelet aggregation and the release of endothelin (ET-1). Abbreviations: Ach, acetylcholine; Bk, bradykinin; *ECE*, endothelin converting enzyme; ET-1, endothelin; Hist, histamine; 5-HT, 5-hydroxytryptamine; pro-ET, pro-endothelin; SP, substance P.

In parallel, the smooth muscle relaxant properties of a class of nitrovasodilators were shown to be endothelium-independent and their action seemed to be due to the liberation of a labile free radical, presumably nitric oxide. In a number of tissue preparations, nitric oxide gas increases soluble guanylyl cyclase (GC) activity to elevate cGMP levels, an effect which could be mimicked using nitric oxide-generating compounds, such as sodium azide, hydroxylamine, sodium nitroprusside and S-nitrosothiols (Arnold *et al.*, 1977; Ignarro *et al.*, 1981). Likewise, agonist stimulated endothelium-dependent relaxations in the rat aorta were associated with activation of GC with a subsequent increase in intracellular cGMP level (Rapoport & Murad, 1983). Around the same time, metabolic balance studies demonstrated that urinary nitrate excretion levels were in excess to dietary ingestion in both conventional and germfree rats as well as in humans, suggesting that nitrate is endogenously synthesized in mammalian cells (Green *et al.*, 1981a,b).

Different pharmacological and chemical approaches were used to provide evidence that EDRF and NO were in fact identical: (i) in cascade bioassay preparations, EDRF released from intact arteries, veins and cultured endothelial cells relaxed detector arteries and increased cGMP content in a similar way as authentic nitric oxide did (Ignarro *et al.*, 1987; Palmer *et al.*, 1987); (ii) the decay of EDRF during passage through the bioassay system resembled that of nitric oxide and the relaxant effect of both compounds was inhibited comparably by haemoglobin, erythrocytes, and methylene blue (Ignarro *et al.*, 1987; Palmer *et al.*, 1987; Gillespie & Sheng, 1988); (iii) similar to EDRF (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986), nitric oxide is stabilized by elimination of superoxide anions employing SOD (Ignarro *et al.*, 1987; Palmer *et al.*, 1987); (iv) Both EDRF and nitric oxide are detected by spectrophotometry after diazotization with sulphanilic acid and cause a characteristic shift in the absorbance spectrum of haemoglobin following their reaction with oxygenated haemoglobin (Ignarro *et al.*, 1987); (v) Final support for the identity of EDRF was provided by chemoluminescence, which is based on the reactivity of nitric oxide or EDRF with ozone to generate a chemoluminescent product. Using this method, Palmer *et al.* (1987) demonstrated that nitric oxide is released from endothelial cells in amounts correlating with relaxations observed in bioassay preparations. Finally, Kelm *et al.* (1988), described the kinetics of nitric oxide with the difference spectrophotometric method and obtained a half-life of 5.6 s, essentially the same as reported previously for EDRF.

Apart from its vasorelaxant properties, NO released from vascular endothelial cells inhibits platelet adhesion and platelet aggregation (Radomski *et al.*, 1987), properties previously attributed to EDRF (Azuma *et al.*, 1986; Förstermann *et al.*, 1989). In spite of striking similarities between EDRF and NO, the chemical identity is still a matter of controversy. For example, the comparable vasorelaxant properties and half life in porcine coronary arteries as well as the ^{35}S efflux from bradykinin-stimulated bovine endothelial cells suggest that S-nitrosocysteine may serve as an alternative candidate for EDRF (Myers *et al.*, 1990; Rubanyi *et al.*, 1991). Indeed, S-nitrosylation is observed with low molecular weight thiols, like cysteine and these potent vasodilators are more stable than NO (Ignarro *et al.* 1981). However, cysteine reduces NO-and EDRF-induced relaxations in the rabbit aorta bioassay system and potentiation of NO-mediated responses occurs only at high concentrations (Liu & Furchgott, 1993; Feelisch *et al.*, 1994) indicating that combination with protein-bound thiol groups is not obligatory for biological activity.

Biosynthesis

The first indications of the source of NO originate from observations that activated murine macrophages, which produced substantial amounts of nitrite and nitrate upon stimulation with lipopolysaccharide (Stuehr & Marletta, 1985), use L-arginine as a substrate and form L-citrulline as a co-product (Hibbs *et al.*, 1987; Iyengar *et al.*, 1987). Porcine endothelial cells, cultured in the absence of L-arginine, showed a decrease in the release of EDRF induced by bradykinin and A23187, which was restored by L-arginine but not D-arginine (Palmer *et al.*, 1988a). Moreover, when incubated with ^{15}N-labelled L-arginine, release of ^{15}NO was observed from immunostimulated macrophages (Marletta *et al.*, 1988) and porcine endothelial cells stimulated with bradykinin (Palmer *et al.*, 1988b). These observations indicated a similar pathway in both cell types and suggest that NO originates from the terminal guanidino nitrogen moiety of L-arginine. Figure 1.4 illustrates the biosynthesis of NO from L-arginine by the enzyme NO-synthase (NOS). The production of NO from the endogenous substrate L-arginine is associated with binding to the prosthetic haeme group and incorporation of molecular oxygen into NO and L-citrulline (Marletta *et al.*, 1988; Leone *et al.*, 1991). This reaction resembles cytochrome P_{450} chemistry, involves a two-step electron transfer which results in the formation of the intermediate hydroxy-L-arginine (L-OHARG), and subsequent oxidation to L-citrulline and NO (Klatt *et al.*, 1993). NOS may use L-OHARG as a substrate and this intermediate has

been proposed to react with NO to form a more stable vasodilator (Zembowicz *et al.*, 1991). In addition, L-OHARG seems to act as a paracrine molecule, since it may enter vascular smooth muscle cells via amino acid transporter mechanisms and is, independently of NOS, oxidized into NO (Schott *et al.*, 1994).

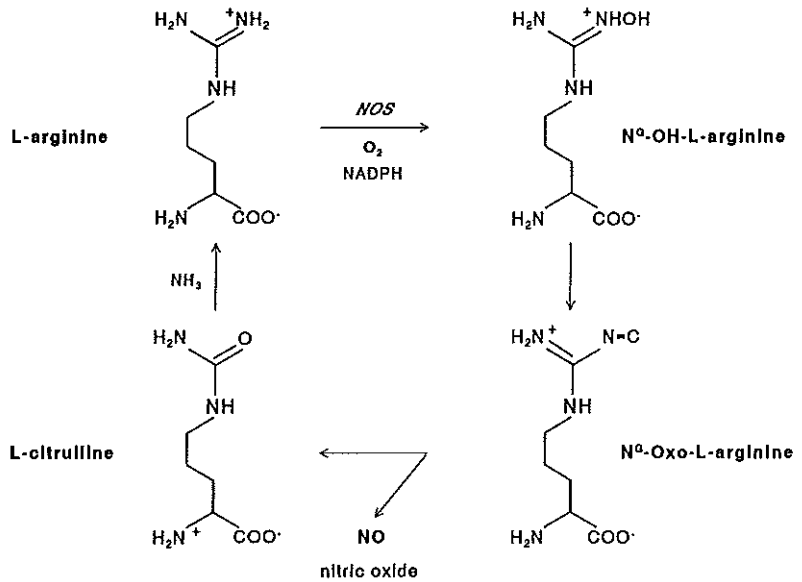

Figure 1.4 Biosynthesis of nitric oxide from L-arginine. L-arginine is bound to NO-synthase (*NOS*) and molecular oxygen (O_2) is incorporated into NO and L-citrulline. The reaction requires NADPH and involves multiple steps. L-citrulline may be reconverted into L-arginine.

Since the initial discovery of acetylcholine-induced endothelium-dependent relaxations, many other vasoactive substances were found to act through the release of NO. These substances include, among others, bradykinin, histamine, adenine nucleotides, thrombin, substance P and 5-hydroxytryptamine (Figure 1.3; for review, see Furchgott & Vanhoutte, 1989). Apart from receptor mediated NO release, hypoxia as well as hydromechanic forces imposed on the blood vessel wall, induce the release

of NO. The tangential component of this force, known as shear stress, causes a deformation of the endothelial lining and the release of factors like NO and prostacyclin. This mechanism may serve to adapt locally to changes in blood flow and pressure and, may account for the basal release of NO (Pohl *et al.*, 1991; Kuchan & Frangos, 1994).

NOS-isoforms

The formation of NO is catalyzed in mammalian cells by a family of at least three isoenzymes, referred to as NOS. The major characteristics of the respective isoforms are summarized in table 1.1. All isoforms are NADPH-dependent haeme containing oxygenases, which require tetrahydrobiopterin and the flavines, FAD and FMN, as cofactor (Bredt *et al.*, 1991; Förstermann *et al.*, 1991). Initially, two distinct forms were described; a constitutive expressed form and an inducible form, denoted as cNOS and iNOS, respectively. The constitutive isoform is mainly present in vascular endothelial cells and neuronal cells, whereas the inducible enzyme is found in activated macrophages, hepatocytes and smooth muscle cells. Subsequently, different NOS isoforms were purified from rat and porcine cerebellum (Bredt & Snyder, 1990; Mayer *et al.*, 1990), bovine endothelium (Förstermann *et al.*, 1991) as well as human brain (Schmidt & Murad, 1991).

Following purification and cloning, the constitutive enzyme appeared to consist of two separate isoforms encoded by different genes (Sessa *et al.*, 1993). Isoform I (ncNOS) was originally purified from rat brain and is a mainly soluble enzyme, although a substantial portion of the enzyme appears to be associated with the endoplasmatic reticulum (Bredt & Snyder, 1990; Hecker *et al.*, 1994a). The deduced amino acid sequence obtained from cDNA clones shows that the molecule has recognition sites for NADPH, FAD, FMN and calmodulin (Bredt *et al.*, 1991). Though its precise function in the regulation of vascular tone is not yet established, this isoform may be involved in neurotransmission in noncholinergic-nonadrenergic (NANC) vasorelaxant nerves surrounding central and peripheral blood vessels (Bredt *et al.*, 1990; Toda *et al.*, 1993).

The inducible isoform (iNOS), a predominantly soluble enzyme, is expressed following stimulation with endotoxin and cytokines (Nathan & Xie, 1994). Unlike the constitutive isoforms, the activity of this isoform is not dependent on intracellular free Ca^{2+} and calmodulin binding. Its induction is associated with the formation of large quantities of NO and its activity is related with macrophage cytotoxicity and vascular

hyporeactivity to vasoconstrictors (Hevel *et al.*, 1991; Julou-Schaeffer *et al.*, 1991; Stuehr *et al.*, 1991).

The second constitutive isoform (isoform III; ecNOS) was identified as a membrane bound enzyme in porcine and human endothelial cells (Förstermann *et al.*, 1991; Hecker *et al.*, 1994b), and its activity is regulated by Ca^{2+} and binding of calmodulin. This isoform produces small amounts of NO and may be the most important isoform involved in the regulation of vascular tone.

Table 1.1 The characteristics of the constitutive and inducible isoforms of NOS. The descriptive nomenclature of the different isoforms is given between parenthesis.

	Isoform		
	I (ncNOS)	II (iNOS)	III (ecNOS)
Source	neuronal cells NANC-neurons astrocytes	macrophages smooth muscle cells	endothelial cells
Type	constitutive	inducible	constitutive
Activation	glutamate	LPS LPS + IL-1 LPS + TNF-α	Ach, ADP, Bk, 5-HT, histamine, shear stress, pressure
NO-release	small	large, continuous	small, pulsatile
Regulation	Ca_i^{2+}/Calmodulin	-	Ca_i^{2+}/Calmodulin
Cofactors	TH_4, FMN, FAD	TH_4, FMN, FAD	TH_4, FMN, FAD
Feedback regulation	inhibitory	inhibitory	inhibitory
Target	guanylyl cyclase	thiols (?)	guanylyl cyclase haemproteins

Abbreviations: Ach, acetylcholine; ADP, adenosine diphosphate; Bk, bradykinin; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; 5-HT, 5-hydroxytryptamine; IL-1, interleukin-1; LPS, lipopolysaccharide; NANC, non-adrenergic non-cholinergic; TH_4, tetrahydrobiopterin; TNF-α, tumor necrosis factor-α. For details, see text.

A common feature of all isoforms is that their activity is regulated by feedback inhibition by NO. In rat cerebellum and endothelial cell homogenates, and macrophages, enzymatic generation of NO and L-citrulline is reduced by exogenous NO (Rogers & Ignarro, 1992; Assreuy *et al.*, 1993; Buga *et al.*, 1993). Preservation of NO using co-incubation with SOD further reduces NOS-activity, whereas inactivation of NO with oxyhaemoglobin can increase NOS activity (Rogers & Ignarro, 1992; Buga *et al.*, 1993). Likewise, agonist-and flow-induced relaxations of isolated arteries are inhibited by NO-donors (Buga *et al.*, 1993), indicating that NOS-activity is regulated by one of its own endproducts. This feedback mechanism is not mediated by c-GMP (Assreuy *et al.*, 1993), but may involve interaction with the prosthetic haem group of NOS and thereby interaction with electron transfer and substrate oxygenation.

Mode of action and metabolism

The lipophilic nature of NO enables a rapid diffusion across cell membranes to adjacent cells and body fluids. It is now generally excepted that in vascular smooth muscle cells endogenous NO as well as NO donors act through stimulation of GC to cause an increase in cGMP. The ability of NO to stimulate GC is readily explained by its high affinity for ferrohaem groups. The mechanism by which cGMP causes smooth muscle relaxation is less well defined and may involve multiple pathways. Both endothelium-dependent (EDRF) and independent vasorelaxations (nitrovasodilators) in rat aorta have been suggested to occur through a dephosphorylation of myosin light chain (Rapoport *et al.*, 1983; McDaniel *et al.*, 1992). Additionally, membrane hyperpolarization has been proposed to participate in smooth muscle relaxation. Though strongly depending on the experimental conditions, nitrovasodilators and NO cause hyperpolarization of vascular preparations (Tare *et al.*, 1990; Garland & McPherson, 1992; Parkington *et al.*, 1993). These hyperpolarizing responses seem to be distinct from those attributed to EDHF (Parkington *et al.*, 1993; Garland *et al.*, 1995). Since hyperpolarization and smooth muscle relaxation are sensitive to high concentrations of K^+ and K^+ channel blockade, the effects involve opening of K^+ channels (Garland & McPherson, 1992). Opening of these channels may be coupled to activation of GC as methylene blue abolished both hyperpolarizations and relaxations of guinea pig uterine arteries (Tare *et al.*, 1990). Furthermore, in rabbit cerebral artery smooth muscle cells hyperpolarization is mimicked by analogues of cGMP and cGMP-dependent protein kinase (Robertson *et al.* 1993). Apart from GC-mediated activation of K^+ channels, NO has been reported to activate Ca^{2+}-

dependent K^+ channels directly in smooth muscle membrane patches from rabbit aorta. The NO-induced relaxations of the isolated rabbit aorta was partially reduced by methylene blue and the remaining component of relaxation was blocked by K^+ channel blockade (Bolotina *et al.*, 1994).

The extremely short biological half-life of NO implicates that it is rapidly inactivated and eliminated from the site of action. It has been speculated that a part of the endogenously released NO is buffered by thiols, especially serume albumin, to serve as a temporarily reservoir for NO (Stamler *et al.*, 1992). However, the common belief is that NO is oxidized to NO_2^- in aqueous solutions containing oxygen (Ignarro *et al.* 1993; Wink *et al.*, 1993). In contrast, in animal and human blood, NO_2^- concentration is within a low nanomolar range and is largely exceeded by the NO_3^- concentration (Castillo *et al.*, 1993; Wennmalm *et al.*, 1993). This apparent difference indicates that oxidation by molecular oxygen to NO_2^- alone can not account for the entire metabolism of NO. Numerous chemical interactions in cells or tissues could explain further elimination, including reaction with oxyhemoglobins, superoxide anion, and other oxygen-derived radicals. Both haemoglobin, oxyhemoglobin, and erythrocytes are known to abolish NO-induced responses in functional studies (see for instance Gillespie & Sheng, 1988). Furthermore, oxyhemoglobin and whole blood catalyze the conversion of NO as well as NO_2^- to NO_3^- (Ignarro *et al.*, 1993; Wennmalm *et al.*, 1993). Hence, endogenously released NO may be oxidized by molecular oxygen to NO_2^-, which is subsequently incorporated in red blood cells to be degraded by oxyhemoglobin to NO_3^-. Alternatively, NO may be taken up by erythrocytes directly to react with oxyhemoglobin yielding NO_3^- and methaemoglobin. Irrespective of the intermediate oxidation to NO_2^-, the final common metabolite of NO seems to be NO_3^-, which is further eliminated via renal excretion.

An additional route of inactivation is through reactivity of NO with free radicals such as superoxide anion (O_2^-). These molecules reduce vasorelant properties attributed to NO in bioassay systems (Gryglewski *et al.* 1986; Rubanyi & Vanhoutte, 1986). The main product of this interaction is peroxinitrite ($ONOO^-$), which, in turn, decomposes to NO_2^- and hydroxyl radicals. Since the formation of the highly reactive intermediate is dependent on the absence of reducing moieties like glutathione, this route of elimination may be of importance under pathophysiological conditions (Moro *et al.*, 1994).

Inhibition of NO-biosynthesis

L-arginine-dependent pathways, such as in macrophage cytotoxicity (Hibbs *et al.*, 1988) and endothelial NO release, can be inhibited by a series of analogues of L-arginine, like N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine (L-NNA). The structure of some of these inhibitors is shown in Figure 1.5. A general feature of all inhibitors is the presence of a modified guanidino group, whereas esterification of L-NNA leads to its water soluble congener N^G-nitro-L-arginine methyl ester (L-NAME). These inhibitors act in an enantio-specific way and their action is usually overcome by an excess of L-arginine, but not D-arginine or L-citrulline.

Binding experiments using tritiated L-NNA have shown that this compound binds to cNOS at a single binding site (Michel *et al.*, 1993; Klatt *et al.*, 1994). To date, it is unclear how NOS is inactivated, but these inhibitors may serve as a false substrate for the enzyme.

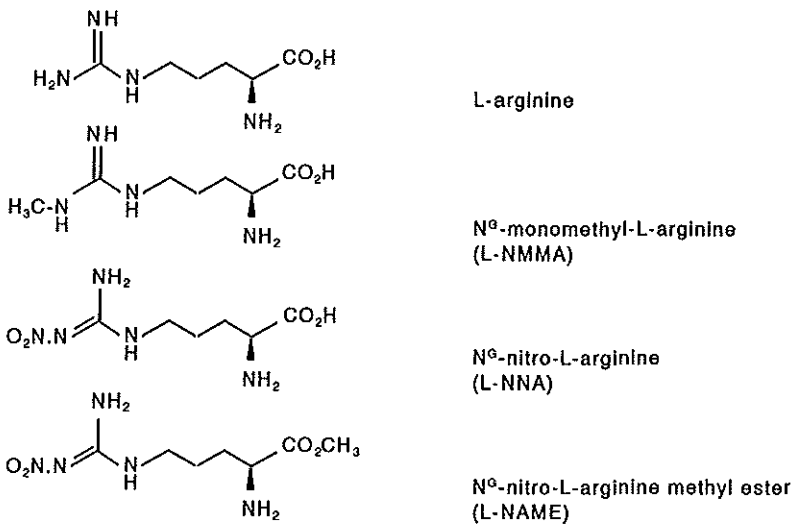

Figure 1.5 Chemical structure of some NOS-inhibitors. N^G-monomethyl-L-arginine, N^G-nitro-L-arginine and its methyl ester L-NAME are derivatives of L-arginine, which contain a modified guanidino moiety. The commonly used abbreviations are denoted between parentheses.

The structural similarities of the NOS inhibitors with the endogenous substrate and the presence of a modified guanidino group, suggest that this moiety accounts for the high affinity for the constitutive enzyme. Furthermore, the electron drawing nitro substituent in L-NNA and L-NAME suggests that conformational changes at the active site of the enzyme may occur (Mülsch & Busse, 1990). Enzyme activity is restored more slowly following inhibition with L-NNA than with L-NMMA which has been related to the conversion of the latter to hydroxylated L-NMMA and L-HOARG, both alternate substrates for NOS (Olken & Marletta, 1993; Klatt *et al.*, 1994). On the other hand, nitro-derivatives may fit better to the active site.

The NOS-inhibitors possess different inhibitory potencies towards the respective isoforms. The nitro-analogues preferentially inhibit the constitutive isoform. For example, L-NNA is 300-fold more potent against brain cNOS than against macrophage iNOS (Furfine *et al.*, 1993). In contrast, L-NMMA is an equipotent inhibitor of both cNOS and iNOS (Gross *et al.*, 1991).Apart from their inhibitory action, some of the compounds possess properties not directly related to NOS. L-NMMA may compete for the cellular L-arginine uptake (Bogle *et al.*, 1992). Since intracellular levels of L-arginine are usually well above the K_m of NOS (Bredt & Snyder, 1990; Förstermann *et al.*, 1991), and L-citrulline can be recycled to L-arginine (Hecker *et al.*, 1990), this may only be relevant during low intracellular L-arginine concentrations, such as following prolonged iNOS activity.

Radioligand binding studies have shown that alkyl esters of L-arginine can compete non-selectively for muscarinic receptors present in vascular tissues. Functionally, antimuscarinic activity could be detected with L-NAME, rather than with L-NMMA and L-NNA, in isolated arterial and visceral smooth muscle preparations (Buxton *et al.*, 1993). The ability of L-NAME to antagonize muscarinic receptor mediated responses may hamper the interpretation of responses attributed to inhibition of NO-biosynthesis.

1.3 Aims of this thesis

The objective of this thesis was to investigate the potential role of endogenous NO in the cardiovascular regulation in general, and in migraine more specifically. For this purpose, separate more specific aims were defined:

1. To investigate the contribution of NO to the regulation of systemic haemodynamics and regional tissue perfusion in anaesthetized animals under different experimental conditions.

2. To investigate the potential role of endogenous NO in hypotensive and hypertensive responses induced by known endothelium dependent agonists, in vivo.

3. To identify tissues containing arteriovenous anastomoses in pigs and to analyze blood flow responses of these vessel segments following inhibition of NO-biosynthesis.

4. To delineate the effects of exogenous NO and calcitonin gene-related peptide on the distribution of blood flow in the porcine carotid circulation.

Chapter 2

Haemodynamic changes and acetylcholine-induced hypotensive responses after N^G-nitro-L-arginine methyl ester in rats and cats

Summary

The basal and agonist-stimulated release of nitric oxide from endothelial cells is inhibited by N^G-nitro-L-arginine methyl ester (L-NAME). In this study, the haemodynamic effects of L-NAME (1, 3, 10 and 30 mg kg^{-1}) and its potential ability to attenuate the hypotensive responses to acetylcholine (0.03, 0.1, 1.0 and 3.0 μg kg^{-1}) have been investigated in anaesthetized rats and cats. In addition, regional tissue blood flows were measured in cats using repeated intracardial injections with radioactive microspheres.

In the rat, L-NAME elicited a dose-dependent pressor effect increasing mean arterial blood pressure from a baseline value of 116 ± 4 mmHg to a maximum of 156 ± 6 mmHg. This pressor response was partly reversed by L-arginine (300 mg kg^{-1}). However, the increase in blood pessure by lower doses (up to 10 mg kg^{-1}) of L-NAME was effectively reversed by L-arginine (1000 mg kg^{-1}).

In cats, L-NAME did not significantly modify systemic haemodynamic variables (heart rate, mean arterial blood pressure, cardiac output, stroke volume or total peripheral resistance). Administration of L-arginine did not cause any significant effect in cats treated with L-NAME, but some decrease in heart rate and increases in cardiac output and stroke volume were observed in the saline-treated group. With the lowest dose, L-NAME did not affect tissue blood flows in the cat, but higher doses (3 and 30 mg kg^{-1}) significantly reduced blood flows to the mesentery, stomach, spleen, intestines, lungs and the total liver. In control (saline-treated) animals, L-arginine (300 mg kg^{-1}) caused a significant increase in blood flow to the heart, mesentery, lungs as well as the total liver, particularly the portal fraction. L-arginine-induced changes in tissue blood flows (mesentery, kidneys, spleen, lungs, total liver and portal blood

Based on: Van Gelderen E.M., Heiligers J.P.C. and Saxena P.R. (1991). Haemodynamic changes and acetylcholine-induced hypotensive responses after N^G-nitro-L-arginine methyl ester in rats and cats. Br. J. Pharmacol. 103; 1899-1904.

flow) in control animals were attenuated in animals treated with L-NAME.

The acetylcholine-induced peak hypotensive response was not reduced in both rats and cats by L-NAME. However, the duration of acetylcholine-induced responses was attenuated by L-NAME in both species. Treatment with L-arginine (10-100 mg kg^{-1}) did not change the acetylcholine-induced hypotension.

These results reveal a marked difference between the haemodynamic effects of L-NAME in rats and cats. In contrast to rats, the role of the L-arginine-NO pathway in the regulation of blood pressure is rather limited in cats, although such a pathway may exist in several tissues. Furthermore, the hypotensive response to acetylcholine in both animal species seems to be largely mediated by NO-independent pathways.

2.1 Introduction

The vasodilator action of acetylcholine in a number of isolated blood vessels depends largely on the release of an endothelium-derived-relaxing factor (EDRF; Furchgott & Vanhoutte, 1989). EDRF has been characterized as being identical to nitric oxide (NO), which is cleaved from L-arginine by the action of the enzyme NOS, present in endothelial cells (Palmer *et al.*, 1987, 1988). Once released, NO stimulates soluble guanylate cyclase to increase cGMP levels (Boulanger *et al.*, 1990; Kelm & Schräder, 1990). The biosynthesis of NO is inhibited in vitro by the arginine analogues, such as N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methylester (L-NAME) (Palmer *et al.*, 1988; Mülsch & Busse, 1990).

The local vasodilation induced by acetylcholine is reported to be inhibited by L-NAME in the rat perfused mesentery (Moore *et al.*, 1990) and by L-NMMA in anaesthetized rats as well as the human forearm arterial bed (Whittle *et al.*, 1989; Vallance *et al.*, 1989). In contrast, L-NAME has not been found to affect the vasodepressor action in conscious rats (Gardiner *et al.*, 1990b). Since most experiments using arginine-analogues have been performed in vitro, we studied the systemic haemodynamic effects of L-NAME as well as its ability to modify the hypotensive responses to acetylcholine in rats and cats. In the latter animal species, tissue blood flows changes following the administration of L-NAME were also investigated.

2.2 Methods

General

Rats: Thirty seven male Wistar-Kyoto rats (body weight 300-350 g) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.). A trachea canula was inserted to facilitate ventilation. Catheters were placed in both jugular veins and the right carotid artery for, respectively, drug administration and blood pressure measurement by a pressure transducer (model P23Ac, Statham Laboratories, Hato Rey, Puerto Rico). Blood pressure was continuously recorded on a polygraph (model 7, Grass Instrument Company, Quincy, MA). Heart rate was derived from the blood pressure recordings. Temperature was maintained at 37 °C using an electric blanket.

Cats: Twelve male or female cats (body weight between 2.8 and 5.0 kg) were anaesthetized with ketamine (12 mg kg^{-1}, i.p.). A trachea canula was inserted for artificial ventilation by a respiratory pump (Loosco, Amsterdam, The Netherlands). Both femoral veins were catheterized for i.v. administration of drugs and blood sampling. Anaesthesia was maintained with sodium pentobarbitone (6 mg kg^{-1}, i.v. bolus, followed by 3 mg kg^{-1} h^{-1}, i.v. infusion). Both femoral arteries were cannulated for blood sampling and measurement of arterial blood pressure with a pressure transducer (model P23Ac, Statham Laboratories, Hato Rey, Puerto Rico). Blood pressure was continuously recorded on a polygraph (model 7, Grass Instrument Company, Quincy, MA). The right atrium was catheterized for microsphere administration. Finally, a flow probe was placed around the ascending aorta; cardiac output was calculated by adding ascending aorta blood flow and myocardial blood flow (see below). Body temperature was maintained at 37°C with an electric blanket and pH and blood gases were kept between normal limits (pO_2, 90-120 mmHg; pCO_2, 25-35 mmHg; pH, 7.35-7.45) by adjusting respiratory rate and tidal volume. A stabilization period of 30-60 min was allowed.

Regional tissue blood flows were measured with the radioactive microsphere technique, by use of the reference blood sample method (Heymann *et al.*, 1977; Saxena *et al.*, 1980). For each measurement, a suspension of about 200,000 microspheres (15 μm diameter, NEN Company, Dreieich, West Germany), labelled with one of the isotopes (^{114}Ce, ^{113}Sn, ^{103}Ru, ^{95}Nb, or ^{46}Sc), was mixed and injected into the left atrium. At the end of the experiment the animals were killed and various tissues were dissected out, weighed and put in vials. The radioactivity in these vials was counted for 5-10 min in a γ-scintillation counter (Packard, Minaxi Autogamma 5000) using suitable

windows for discriminating the different isotopes. Regional blood flows and cardiac output were calculated with a set of computer programs especially designed for the radioactive microsphere technique (Saxena *et al.*, 1980).

Experimental protocol

Rats: After a stabilization period each rat received five increasing i.v. doses of acetylcholine (0.03, 0.1, 0.3, 1.0 and 3.0 μg kg^{-1}) at intervals of 3-5 min. Peak hypotensive responses to acetylcholine and its duration of action, i.e. the time needed for 50% recovery of the effect, were recorded. Due to the short-lasting action of acetylcholine, duration of action could only be assessed for the three highest doses. The rats were then divided into 4 groups which received either saline (4 times 1 ml kg^{-1}; n=9), L-NAME (1, 3, 10 and 30 mg kg^{-1}; n=12), L-arginine (10, 30 and 100 mg kg^{-1}; n=7) or phenylephrine (40, 120, 220 and 330 μg kg^{-1} h^{-1}; n=9). In most of the animals (for n, see results), the different doses of saline or drugs, administered every 15-20 min, were followed by the five doses of acetylcholine. The animals which had received L-NAME were administered L-arginine (300 mg kg^{-1}) after the last injection of acetylcholine. An additional group of animals (n=6) was treated with L-NAME (1, 3 and 10 mg kg^{-1}) followed by L-arginine (1000 mg kg^{-1}).

The dose range of phenylephrine was chosen to induce sustained increases in blood pressure similar to that induced with L-NAME. The magnitude and duration of hypotensive responses to acetylcholine after the different treatments were compared to the respective controls.

Cats: A similar protocol was followed in cats as in rats for L-NAME (1, 3, 10 and 30 mg kg^{-1}) and saline. After the first series of acetylcholine injections (0.03, 0.1, 0.3, 1.0 and 3.0 μg kg^{-1}) at baseline, the first batch of microspheres was injected in each animal to determine baseline values of cardiac output and regional haemodynamic variables. The animals then received either four doses of L-NAME (1, 3, 10 and 30 mg kg^{-1}, n=7) or saline (1 ml kg^{-1}, n=5) at intervals of 20 minutes. Ten minutes after each dose (except after 10 mg kg^{-1} L-NAME and the third dose of saline), a batch of microspheres was injected. Finally, a bolus injection of 300 mg kg^{-1} L-arginine was administered, followed ten minutes later by the last batch of microspheres for tissue blood flow measurements.

Data presentation and analysis

All data in the text are mentioned as mean ± s.e.mean. The values after the different treatments were compared to the baseline values by using Duncan' new multiple range test once a parametric two-way analysis of variance (randomized block design) had revealed that the samples represented different populations. The significance of the changes induced by L-NAME, L-arginine or phenylephrine as compared to the respective saline treatment was tested by an unpaired Student *t*-test. A P-value of 0.05 or less was considered statistically significant.

Drugs

The drugs used in this study were: L-arginine hydrochloride, N^G-nitro-L-arginine methylester (both drugs: Sigma, St Louis, U.S.A.), ketamine hydrochloride (A.U.V., Cuyk, The Netherlands), acetylcholine chloride, phenylephrine hydrochloride (both from the Department of Pharmacy, Erasmus University, Rotterdam, The Netherlands), sodium pentobarbital (Sanofi BV, Maassluis, The Netherlands). All drugs were dissolved in sterile saline. The doses mentioned in the text refer to the respective salts.

2.3 Results

Rats

Haemodynamic effects

Both mean arterial blood pressure and heart rate remained relatively stable in the rats receiving either saline or L-arginine injections. Both L-NAME and phenylephrine elicited pressor responses; from the baseline values of 116 ± 4 and 93 ± 4 mmHg, respectively, blood pressure increased dose-dependently to a maximum of 156 ± 6 and 135 ± 5 mmHg, respectively, with the highest doses. Heart rate decreased significantly compared to baseline values with L-NAME, but the decreases were not different from those seen in saline-treated animals. Phenyleprine did not change heart rate (Figure 2.1). The increase in blood pressure induced by L-NAME (30 mg kg^{-1}) could be partly reversed by the administration of L-arginine (300 mg kg^{-1}) (Figure 2.1).

In an additional group of 6 animals which received L-NAME up to 10 mg kg^{-1}, blood pressure increased significantly from 93 ± 2 to a maximum of 142 ± 4 mmHg and heart rate decreased significantly from 336 ± 11 to 282 ± 13 beats min^{-1}. L-arginine reversed the L-NAME-induced increase in blood pressure, which returned

to a value of 78 ± 9 mmHg ($p<0.05$, versus L-NAME 10 mg kg^{-1}). However, the bradycardia induced by L-NAME remained unchanged after L-arginine (288 ± 13 beats min^{-1}).

Acetylcholine responses

Acetylcholine (0.03-3.0 μg kg^{-1}) elicited a dose-dependent fall in mean arterial blood pressure in rats; before any treatment the highest dose induced a fall in blood pressure of 52 ± 11 mmHg (n=25). The calculated dose inducing a 40 mmHg decrease in mean arterial blood pressure (D_{40}) was 0.8 ± 0.1 μg kg^{-1} (n=25). Treatment of rats with the four doses of saline did not influence the hypotensive responses to acetylcholine (Figure 2.2). Compared to the rats treated with saline, L-NAME (3 and 10 mg kg^{-1}) increased the hypotensive effects of 0.3 to 3.0 μg kg^{-1} acetylcholine. The vasodilator response to acetylcholine was also potentiated during infusions of phenylephrine (220 and 330 μg kg^{-1} h^{-1}). Pretreatment with L-arginine (10-100 mg kg^{-1}) did not change the acetylcholine-induced hypotension (Figure 2.2).

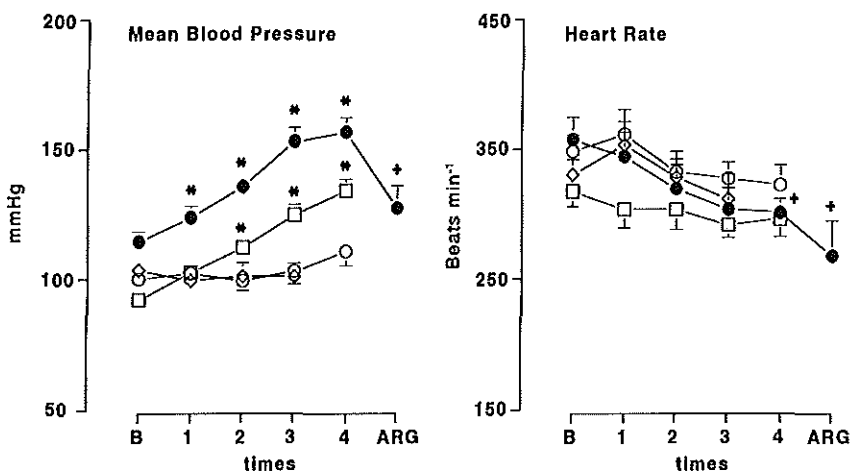

Figure 2.1. Mean arterial blood pressure (MAP) and heart rate in rats at baseline (B) and after treatment with four consecutive doses of saline (1 ml kg^{-1} each, n=9; o-o), L-NAME (1, 3, 10 and 30 mg kg^{-1}, n=12; ●-●), phenylephrine (40, 120, 220 and 330 μg kg^{-1} h^{-1}, n=9; □-□) or L-arginine (10, 30 and 100 mg kg^{-1}, n=7; ◊-◊). L-arginine (ARG; 300 mg kg^{-1}) was injected in the animals that had received L-NAME. Data are given as mean ± s.e.mean. *, $P<0.05$ versus saline; +, $P<0.05$ versus baseline values.

The duration of the depressor response to acetylcholine, assessed as the time needed for 50% recovery, remained unchanged after treatments with either saline, L-arginine or phenylephrine. However, there was some significant reduction in the duration of action of acetylcholine (0.3 and 1.0 μg kg-[1]) by L-NAME (Figure 2.3).

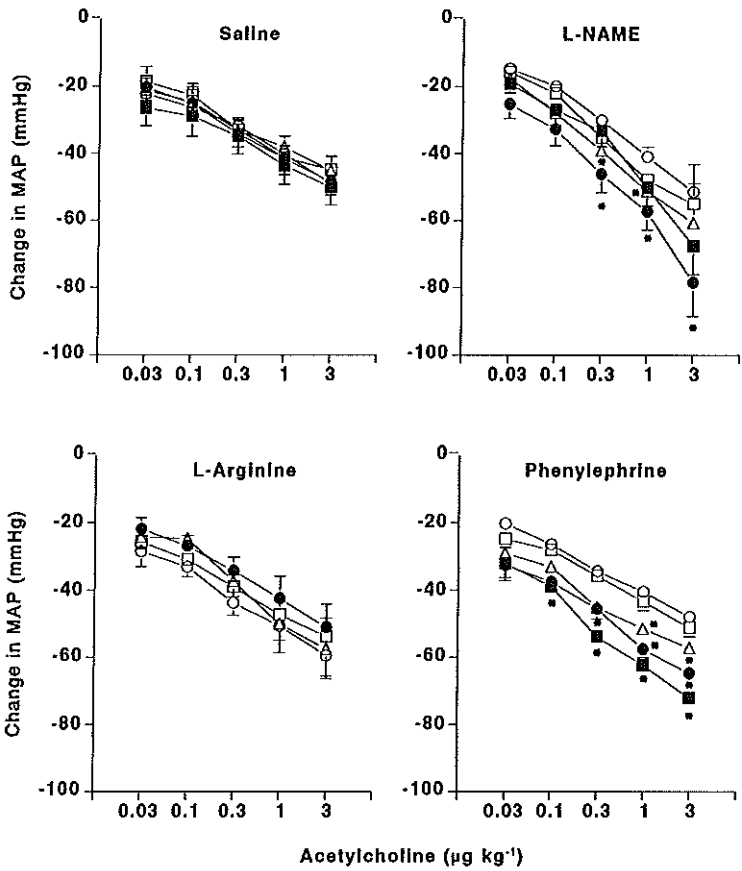

Figure 2.2 Decreases in mean arterial blood pressure (MAP) by acetylcholine (0.03-3.0 μg kg^{-1}) in rats before (control; ○) and after treatments with saline (1 ml kg^{-1} four times, n=8), L-NAME (1, 3, 10 and 30 mg kg^{-1}, n=8), phenylephrine (40, 120, 220 and 330 μg kg^{-1} h^{-1}, n=7) or L-arginine (10, 30 and 100 mg kg^{-1}, n=7). The increasing doses in each case are represented by the symbols □, △, ● and ■, respectively. Data are given as mean ± s.e.mean. *, P<0.05 versus saline.

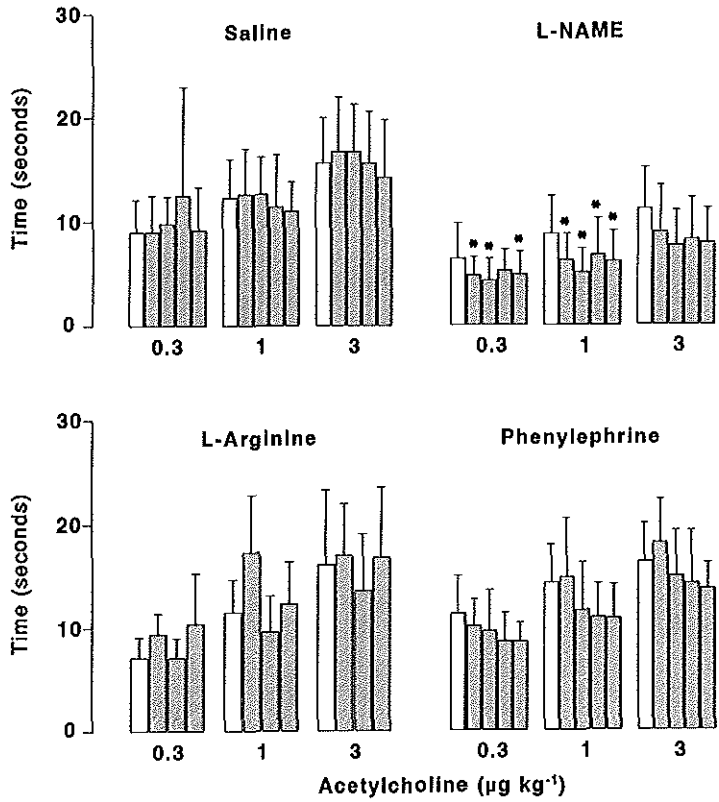

Figure 2.3 Time needed for 50% recovery in the hypotensive action of acetylcholine (0.3, 1 and 3 µg kg^{-1}) in rats treated with saline (1 ml kg^{-1} four times, n=8), L-NAME (1, 3, 10 and 30 mg kg^{-1}, n=8), L-arginine (10, 30 and 100 mg kg^{-1}, n=7) or phenylephrine (40, 120, 220 and 330 µg kg^{-1} h^{-1}, n=7). In each set of panels, the 5 columns (4 in case of L-arginine) represent, sequentially, the responses before and after the increasing doses of saline or drugs. Data are given as mean ± s.e.mean. *, $P<0.05$ versus saline.

Cats

Haemodynamic effects

The baseline values of the systemic and regional haemodynamic variables in the two groups of cats receiving, respectively, saline and L-NAME, both followed by L-arginine (300 mg kg^{-1}) are listed in table 2.1. No significant differences were observed between the two groups.

Figure 2.4 depicts the percent changes in the systemic haemodynamic variables caused by saline (three injections) and L-NAME (1, 3 and 30 mg kg^{-1}). Treatment with saline did not cause any significant change other than a small decrease in heart rate. Similarly, the systemic haemodynamic effects of L-NAME in the cat were not marked.

Table 2.1 Baseline values of systemic haemodynamic variables and organ blood flow in anaesthetized cats treated with saline (n=5) or L-NAME (n=7).

	Saline	L-NAME
Systemic haemodynamic variables		
MAP *(mmHg)*	101.2 ± 6.1	95.1 ± 5.6
HR *(beats min^{-1})*	153.0 ± 4.9	143.3 ± 14.6
SV *(ml)*	3.3 ± 0.3	3.9 ± 0.8
CO *(ml min^{-1})*	503.8 ± 42.1	512.7 ± 76.9
TPR *(mmHg l^{-1} min^{-1})*	203.5 ± 11.4	214.4 ± 29.0
Organ blood flow (ml min^{-1})		
Heart	13.5 ± 5.0	10.1 ± 1.5
Brain	8.5 ± 1.8	7.1 ± 1.1
Lungs	39.9 ± 5.7	39.8 ± 5.2
Stomach	8.0 ± 2.1	7.2 ± 1.7
Intestine	27.8 ± 6.0	25.6 ± 3.3
Spleen	14.0 ± 4.6	7.1 ± 1.6
Mesentery	7.6 ± 2.6	5.2 ± 1.1.
Portal	68.7 ± 16.4	58.4 ± 9.5
Total liver	86.2 ± 23.7	71.0 ± 8.9
Kidneys	39.2 ± 5.9	38.5 ± 4.4
Muscle	3.8 ± 0.9	3.4 ± 0.4
Skin	1.4 ± 0.2	1.5 ± 0.4

Abbreviations: MAP, mean arterial blood pressure; HR, heart rate; SV, stroke volume; CO, cardiac output; TPR, total peripheral resistance.

Although there was a small increase in total peripheral resistance (29 ± 5%) after 3 mg kg^{-1} L-NAME, these changes were only slightly more than those with the corresponding dose of saline (12 ± 5%). In the saline group, L-arginine decreased heart rate significantly (21 ± 3%), but cardiac output increased (12 ± 3%) due to an increase in stroke volume (42 ± 7%) (Figure 2.4). Administration of L-arginine did not cause any significant effect in cats treated with L-NAME, but the changes observed in the saline group appeared to be blunted (Figure 2.4).

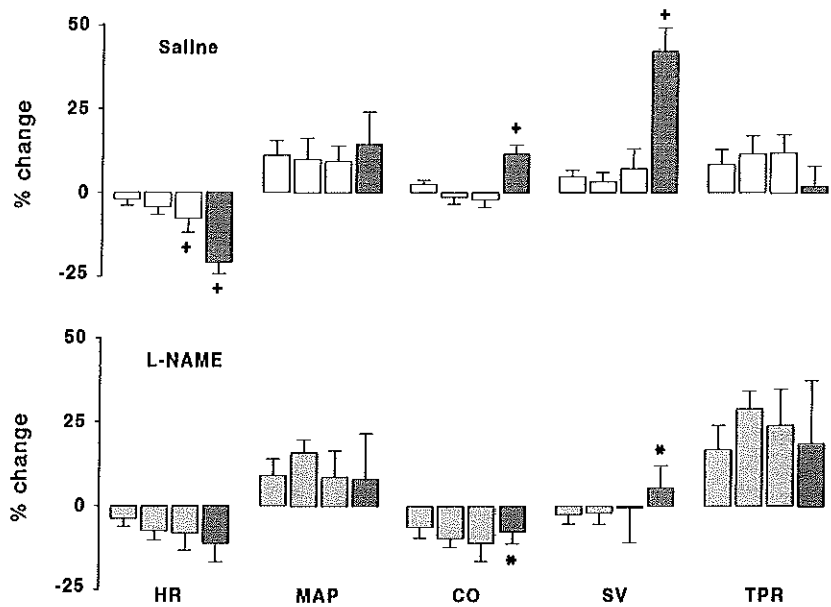

Figure 2.4 Percentage changes from baseline in systemic haemodynamic values in cats treated with saline (1 ml kg^{-1} three times, n=5) or L-NAME (1, 3 and 30 mg kg^{-1}, n=7). Data after the 3rd dose of saline or L-NAME (10 mg kg^{-1}), where no microspheres were given (see Methods), were not collated. In each set of panels, the first three columns represent, sequentially, the increasing doses of saline or L-NAME, while the fourth column represents L-arginine (300 mg kg^{-1}). Abbreviations: HR, heart rate; MAP, mean arterial blood pressure; CO, cardiac output; SV, stroke volume; TPR, total peripheral resistance. Data are given as mean ± s.e.mean. *, $P<0.05$ versus saline; +, $P<0.05$ versus baseline.

The effects of L-NAME (1, 3 and 30 mg kg^{-1}) on tissue blood flows in the cat are shown in Figures 2.5.1 and 2.5.2. With the lowest dose no changes were observed, but the higher doses of L-NAME significantly reduced blood flows to several tissues including the mesentery, stomach, spleen, intestines, lungs and the total liver. L-arginine (300 mg kg^{-1}) injected into the control (saline-treated) animals resulted in a significant increase in blood flow to the heart, mesentery, lungs as well as the total liver, particularly its portal fraction (Figure 2.5.1 and 2.5.2). The changes induced by L-arginine in animals treated with L-NAME were significantly different from the changes in saline treated animals in the mesentery, kidneys, spleen, lungs as well as the total liver and portal blood flow (Figure 2.5.1 and 2.5.2).

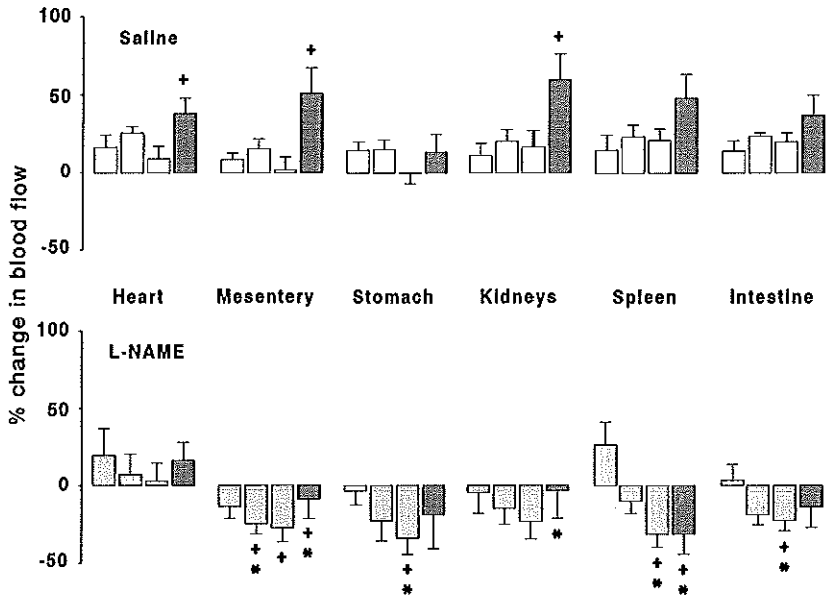

Figure 2.5.1 Percentage changes from baseline in tissue blood flows in cats treated with saline (1 ml kg^{-1} three times, n=5) or L-NAME (1, 3 and 30 mg kg^{-1}, n=7). As mentioned in Methods, tissue blood flows were not measured after the 3rd dose of saline or L-NAME (10 mg kg^{-1}). In each sets of panels, the first three columns, sequentially, represent the increasing doses of saline or L-NAME, while the fourth column represents L-arginine (300 mg kg^{-1}). Data are given as mean ± s.e.mean. *, $P<0.05$ versus saline; +, $P<0.05$ versus baseline.

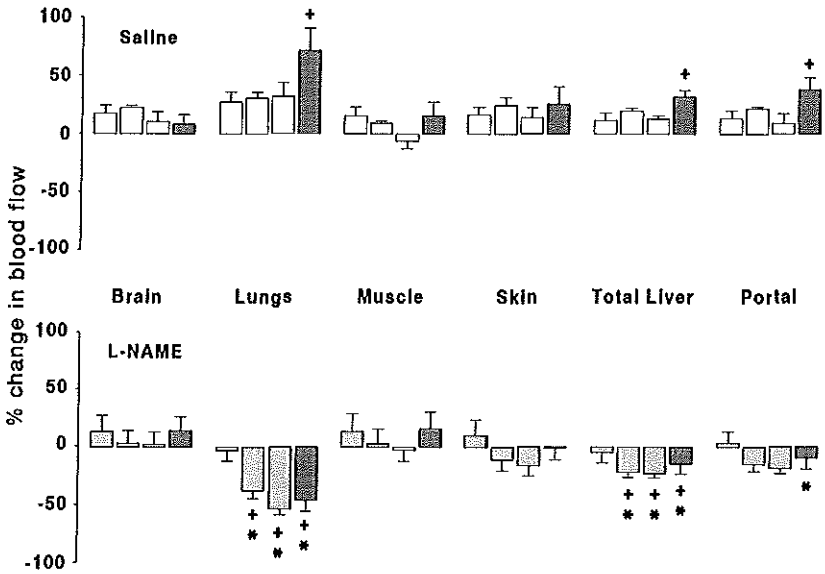

Figure 2.5.2 Percentage changes from baseline in tissue blood flows in cats treated with saline (1 ml kg^{-1} three times, n=5) or L-NAME (1, 3 and 30 mg kg^{-1}, n=7). As mentioned in Methods, tissue blood flows were not measured after the 3rd dose of saline or L-NAME (10 mg kg^{-1}). In each sets of panels, the first three columns, sequentially, represent the increasing doses of saline or L-NAME, while the fourth column represents L-arginine (300 mg kg^{-1}). Data are given as mean ± s.e.mean. *, $P<0.05$ versus saline; +, $P<0.05$ versus baseline.

Acetylcholine responses

Acetylcholine induced a dose-dependent hypotensive effect in anaesthetized cats in doses ranging from 0.03 to 3.0 μg kg^{-1}. The fall in mean arterial blood pressure after the highest dose was 62 ± 5 mmHg (n=9). The calculated dose of acetylcholine inducing a 40 mmHg decrease in mean blood pressure (D_{40}) was 0.11 ± 0.04 μg kg^{-1} (n=9). L-NAME (1, 3, 10 and 30 mg kg^{-1}) did not significantly affect the fall in blood pressure induced by acetylcholine (Figure 2.6). However, L-NAME (from 3 mg kg^{-1} and higher) was able to reduce the duration of action of acetylcholine; the time needed for 50% recovery in the acetylcholine responses was clearly reduced (Figure 2.7).

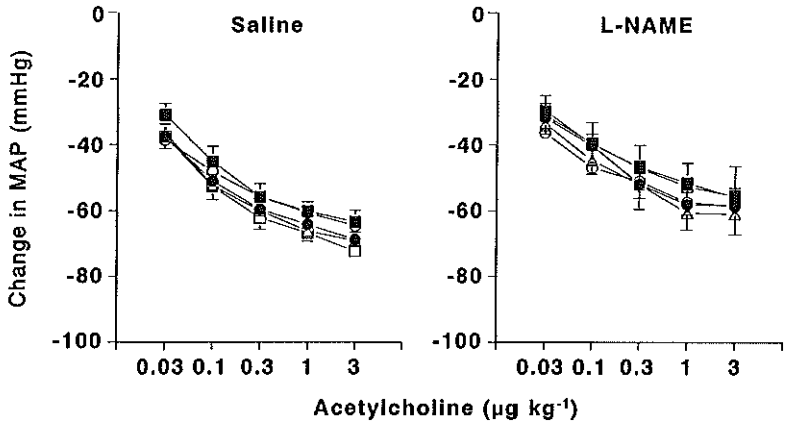

Figure 2.6 Decreases in mean arterial blood pressure (MAP) by acetylcholine (0.03-3.0 µg kg^{-1}) in cats before (control;○) and after treatments with saline (1 ml kg^{-1} four times, n=5) or L-NAME (1, 3, 10 and 30 mg kg^{-1}, n=6). The increasing doses in each case are represented by the symbols □, △, ● and ■, respectively. Data represent mean ± s.e.mean. No significant differences between saline and L-NAME experiments were noticed.

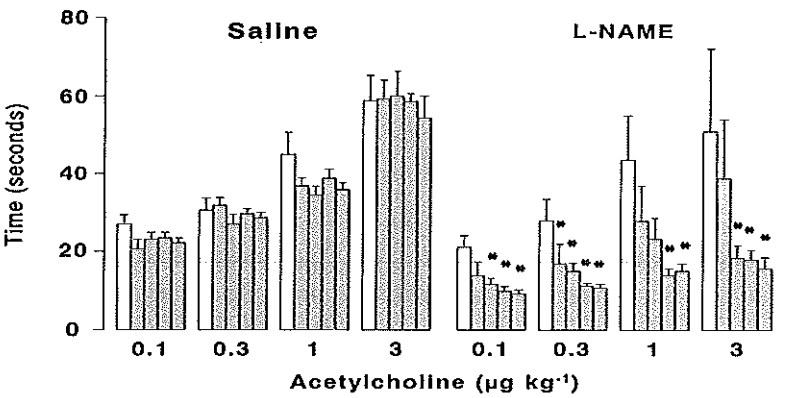

Figure 2.7 Time needed for 50% recovery in the hypotensive action of acetylcholine (0.3-3.0 µg kg^{-1}) in cats treated with saline (1 ml kg^{-1} four times, n=5) or L-NAME (1, 3, 10 and 30 mg kg^{-1}, n=6). In each set of panels, the 5 columns represent, sequentially, the responses before and after increasing doses of saline or L-NAME. Data are given as mean ± s.e.mean. *, $P<0.05$ versus saline.

2.4 Discussion

Haemodynamic responses

Our findings that L-NAME, when injected in pentobarbitone anaesthetized rats, induced a significant dose-dependent increase in blood pressure and reversal by L-arginine is consistent with results found in conscious as well as anaesthetized rats (Gardiner *et al.*, 1990a,b,c; Rees *et al.*, 1990). However, the concomitant fall in heart rate reported in anaesthetized rats by Rees *et al.* (1990) was comparatively less marked with L-NAME in our experiments as heart rate also decreased in animals treated with saline only. Administration of L-arginine, particularly when its dose was higher (100 fold) than that of L-NAME (see also Rees *et al.*, 1990), reversed the increases in blood pressure induced by L-NAME.

In contrast to rats, surprisingly, no significant changes in mean arterial blood pressure were found with four consecutive doses of L-NAME (1, 3, 10 and 30 mg kg^{-1}) in cats anaesthetized with pentobarbital and ketamine (initially). This inability of L-NAME to elicit hypertension has also been observed in cats anaesthetized with α-chloralose and pentobarbital (R. Saxena, E. Scheffers & R. Shukla, unpublished). Though L-NAME had little overall effect on haemodynamics, the drug did elicit a dose-dependent and significant decrease in blood flow to and, therefore, an increase in vascular resistance in several tissues (lungs, liver, stomach, small intestine and mesentery), suggesting an inhibition of the release of NO by L-NAME in these organs. Administration of L-arginine in cats, treated with L-NAME, did not reverse the decrease in blood flow induced by L-NAME. In saline treated animals, however, L-arginine revealed a significant increase in blood flow in the lungs, mesentery, liver and several other organs, including the heart and the kidneys. The above results suggest that in cats there seems to be a difference in the basal release of NO; some organs, like the lungs, liver and mesentery, show more basal release than other tissues like the heart and kidneys. The findings that neither L-NAME nor L-arginine changed the mean arterial blood pressure and that L-NAME induced a decrease in blood flow in only a few organs suggest little contribution of the L-arginine-NO pathway to blood pressure regulation in the anaesthetized cat.

Although L-arginine increased blood flow in several organs, the total peripheral resistance did not change. This can be due to a concomitant increase in cardiac output, following an enhanced stroke volume due to an improved cardiac filling. Gardiner *et al.* (1990c) suggest that the decrease in cardiac output by L-NAME in rats could be

due to a direct negative inotropic action or due to a coronary vasoconstriction. Indeed, L-NAME can induce coronary vasoconstriction in conscious rabbits (Humphries *et al.*, 1991). Our experiments in cats, however, show that despite a significant increase in myocardial blood flow by L-arginine, L-NAME neither decreased coronary artery blood flow nor decreased cardiac output. This again suggests that, unlike in the rat, there is little spontaneous release of NO from the cat heart.

Acetylcholine responses

There seems to be little doubt that the vascular smooth muscle relaxation in vitro or local vasodilation in several vascular beds following acetylcholine administration in different species is largely dependent on the release of NO and is, consequently, inhibited by drugs like L-NAME (Aisaka *et al.*, 1989; Furchgott & Vanhoutte, 1989; Vallance *et al.*, 1989; Whittle *et al.*, 1989; Kontos *et al.*, 1990; Moore *et al.*, 1990). However, it has also been reported that the hypotensive response to acetylcholine in conscious rats is not amenable to blockade by L-NAME (Gardiner *et al.*, 1990b). Our results, both in the anaesthetized rats and cats, are in agreement with the latter findings. Indeed, the hypotensive effect of acetylcholine was even potentiated in the rat after treatment with 10 mg kg^{-1} L-NAME. This apparent potentiation was due to the increase in arterial pressure by L-NAME, since it was also observed after phenylephrine infusions in the rat, but not during L-NAME treatment in the cat where arterial pressure was not affected by the drug. Therefore, it seems likely that the magnitude of the hypotension elicited by acetylcholine *in vivo* is not dependent on the release of NO. It may, however, be noted that the highest dose of L-NAME (30 mg kg^{-1}) did not further increase the depressor responses to acetylcholine in rats; on the contrary the magnitude of the responses were not significantly different from those in saline-treated animals (see Figure 2.2), suggesting that there may be some inhibition of the acetylcholine depressor effects in rats at the highest dose of L-NAME.

Despite little changes in the magnitude of the hypotensive response to acetylcholine, there was a significant reduction in its duration by L-NAME in both cats and rats; phenylephrine infusion in the rat had no effect. In contrast to experiments in guinea pigs with L-NMMA (Aisaka *et al.*, 1989), L-arginine did not prolong the duration of the acetylcholine response in our experiments. Nevertheless, our findings do support the suggestions of Aisaka *et al.* (1989) that the first phase of the depressor response to acetylcholine is NO-independent. It is tempting to speculate that the second phase is, in part, due to a hyperpolarization of the vascular smooth muscle resulting

from the release of an endothelium-dependent-hyperpolarizing-factor (EDHF). Indeed, in dog mesenteric arteries the hyperpolarization induced by acetylcholine, in contrast to the relaxation, seems to be NO-independent, since oxyhaemoglobin and methylene blue only prevented the latter (Komori *et al.*, 1988). However, in guinea pig isolated uterine arteries L-NMMA blocked both hyperpolarization and relaxation responses to acetylcholine suggesting that EDHF may be identical to NO in this species (Tare *et al.*, 1990).

Lastly, it may be mentioned that acetylcholine, when applied topically, induces cerebral dilatation in cats by releasing an EDRF-like substance (Kontos *et al.*, 1990). Our experiments, however showed no significant increase in brain blood flow by L-arginine or a decrease by L-NAME. These findings may indicate that, unlike large conducting vessels, there is only a limited contribution of EDRF/NO to cerebral blood flow regulation.

In conclusion, the present in vivo experiments reveal a marked difference between the effects of L-NAME in rats and cats; in contrast to rats, the contribution of the L-arginine-NO pathway to blood pressure regulation in the cat seems to be very limited, although such a pathway seems to exist in several tissues. In addition, L-NAME does not affect the magnitude of hypotensive response to acetylcholine in either species, but reduces its duration.

Chapter 3

Effect of N^G-nitro-L-arginine methyl ester on the hypotensive and hypertensive responses to 5-hydroxytryptamine in the pithed rat

Summary

The haemodynamic effects of the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 1, 3 and 10 mg kg^{-1}) were studied in pithed rats. Furthermore, we evaluated the ability of this compound to modulate blood pressure responses to 5-hydroxytryptamine (5-HT), 5-carboxamidotryptamine (5-CT) and (1-(2,5-dimethoxy-4-iodophenyl)-2-amino propane (DOI). L-NAME elavated mean arterial blood pressure from 59±1 mmHg to a maximum of 126±6 mmHg, without changing heart rate. A comparable increase in blood pressure was observed with repeated infusions of phenylephrine (160, 240 and 400 μg kg^{-1} h^{-1}); maximum 124±5 mmHg from baseline 54±2 mmHg. In contrast to L-NAME, phenylephrine caused tachycardia with the highest dose. Blood pressure responses to either L-NAME or phenylephrine were unaffected by pretreatment with the $5\text{-}HT_2$-receptor antagonist ketanserin (0.1 mg kg^{-1}).

The hypotensive responses to 5-HT in the presence of ketanserin were augmented by L-NAME as well as phenylephrine infusion. L-NAME also tended to prolong the duration of the response to 5-HT. Likewise, the hypotensive responses to 5-CT were potentiated. The magnitude of hypertensive responses to 5-HT was unaffected by either L-NAME or phenylephrine. However, in contrast to phenylephrine, L-NAME prolonged the duration of these responses. The magnitude and duration (middle dose only) of the hypertensive responses to DOI were augmented by L-NAME, whereas phenylephrine was ineffective.

These results suggest that L-NAME increases blood pressure, probably by inhibiting the basal release of NO in animals with a low vascular tone. However, the

Based on: Van Gelderen E.M. and Saxena P.R. (1992). Effect of N^G-nitro-L-arginine methyl ester on the hypotensive and hypertensive responses to 5-hydroxytryptamine in pithed rats. Eur. J. Pharmacol. 222; 185-191.

hypotensive responses to 5-HT and 5-CT seem to be largely independent of NO-release by the endothelium, but the hypertensive responses to 5-HT and DOI appear to be limited by release of NO in the pithed rat.

3.1 Introduction

The vascular endothelium has been shown to regulate vascular tone by releasing a number of vasoactive substances. One of these substances is nitric oxide (NO), which is formed from L-arginine by the enzyme NO-synthase (Palmer *et al.*, 1987, 1988). The production of NO can be inhibited by a number of analogues of L-arginine, including N^G-nitro-L-arginine (L-NNA) (Dubbin *et al.*, 1990; Mülsch & Busse, 1990). Its methyl ester, N^G-nitro-L-arginine methyl ester (L-NAME) increases blood pressure in both conscious and anaesthetized rats suggesting a NO-dependent vasodilator tone in these animals (Gardiner *et al.*, 1990; Rees *et al.*, 1990; chapter 2, this thesis).

5-Hydroxytryptamine (5-HT) induces a variety of vascular effects, which may partially depend on intact endothelial function (Cocks & Angus, 1983; Saxena & Villalón, 1990). Thus, the removal of endothelium from the rat perfused arteries augments the contraction (mesenteric; Dohi & Lüscher, 1991) or abolishes the vasodilatation (coronary; Mankad *et al.*, 1991) caused by 5-HT. Moreover, treatment with NO-synthase inhibitors inhibits the 5-HT-induced vasodilatation in the perfused, endothelium-intact coronary and renal arteries of the rat (Mankad *et al.*, 1991; Verbeuren *et al.*, 1991). Despite the undeniable evidence for a role for NO in the vasorelaxant responses to 5-HT *in vitro*, there is no information if the release of NO is important in such responses *in vivo*. Indeed, contrary to the well-known mediation of acetylcholine-induced vasodilatation by NO (see Furchgott & Vanhoutte, 1989), we have recently reported that the peak magnitude of hypotensive responses to acetylcholine is not reduced in the anaesthetized rat or cat by L-NAME (chapter 2, this thesis). Therefore, we have now investigated the putative role of NO in the hypotensive and hypertensive responses to 5-HT, before and after L-NAME, in the pithed rat. The rats were pithed to avoid the initial hypotensive response, initiated as a result of activation of Bezold-Jarisch reflex (see Saxena & Villalón, 1990). For comparison, the 5-HT_1-like receptor agonist 5-carboxamidotryptamine (5-CT) and the relative selective

5-$HT_{1C/2}$ receptor agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) have also been used. According to the current view on receptor classification, 5-HT_{1C} receptors have been reclassified as 5-HT_{2C} (Hoyer *et al.*, 1994).

3.2 Methods

General

Male Wistar rats, body weight 350-400 g, were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.). A tracheal cannula was used to allow artificial respiration by a respiratory pump (Loosco, Amsterdam, The Netherlands). Subsequently, the animals were pithed by introducing a stainless-steel rod in the spinal canal via the orbit. Catheters were placed in the right jugular vein and carotid artery for, respectively, drug administration and blood pressure measurement using a pressure transducer (model P23Ac, Statham Laboratories, Hato Rey, Puerto Rico). Blood pressure was continuously recorded on a polygraph (model 7, Grass Instrument Company, Quincy, MA). Heart rate was calculated from the blood pressure recordings. Another catheter was placed in the left femoral vein for the infusion of phenylephrine, which was used as a 'control' for L-NAME. Phenylephrine induces similar changes in carotid, hindquarter, mesenteric and renal conductances as L-NAME (Fozard & Part, 1992).

Body temperature was maintained at 37°C with an electric blanket and arterial blood pH and gases were kept within normal limits (pO_2, 90-120 mm Hg; pCO_2, 25-35 mm Hg; pH, 7.35-7.45) by adjusting respiratory rate and tidal volume. A stabilization period of at least 30 min was allowed.

Experimental protocols

Hypotensive responses

5-HT induces a biphasic blood pressure response consisting of hypotension and hypertension in the pithed rat (Fozard & Leach, 1968). Therefore, to prevent the hypertensive responses the rats (n=29) were first treated with ketanserin (0.1 mg kg^{-1}). This dose of ketanserin effectively blocks 5-HT_2 receptor-mediated hypertension without much affecting the responses to methoxamine in the pithed rat (Kalkman *et al.*, 1982). About 10-15 min later, the animals received four increasing i.v. doses of 5-HT (0.1, 0.3, 1.0 and 3.0 μg kg^{-1}) at intervals of approximately 5 min. The peak

hypotensive response to each dose of 5-HT was recorded. Due to the short-lasting action of 5-HT, the duration of action expressed as the time needed for 50% recovery of the effect ($T_{1/2}$) was assessed only for the two highest doses. The animals were subsequently divided into three groups which received i.v. either saline (3 times 0.5 ml kg^{-1}; n=7), L-NAME (1, 3, 10 mg kg^{-1}; n=13) or phenylephrine (160, 240 and 400 µg kg^{-1} h^{-1}; n=9); the latter was used to match the hypertensive responses associated with L-NAME (see chapter 2, this thesis). Ten minutes after each treatment, the injections of 5-HT were repeated. In order to limit the duration of the experiments not all doses of 5-HT were injected in each animal after each dose of L-NAME, phenylephrine or saline.

In four separate groups of pithed animals the hypotensive response to cumulative doses of 5-CT (0.01 to 3.0 µg kg^{-1} i.v.) was studied after pretreatment with either saline (0.5 ml kg^{-1}, i.v.; n=5) or L-NAME (1, 3 and 10 mg kg^{-1}, i.v.; n=5 each).

Hypertensive responses

Thirty-one rats received three increasing i.v. doses of 5-HT (3, 10 and 30 µg kg^{-1}) at approximately five minutes intervals. The peak hypertensive response to and the duration of action ($T_{1/2}$) of each dose of 5-HT was recorded. Thereafter, the animals were also divided into three groups which received i.v. either saline (3 times 0.5 ml kg^{-1}; n=8), L-NAME (1, 3 and 10 mg kg^{-1}; n=15) or phenylephrine (160, 240 and 400 µg kg^{-1} h^{-1}; n=8). Ten minutes later, the doses of 5-HT injections were repeated. Again, in order to limit the duration of the experiments not all doses of 5-HT were injected in each animal after each dose of L-NAME, phenylephrine or saline.

In two separate groups of animals, DOI (3, 10 and 30 µg kg^{-1}) was injected i.v. before and after either L-NAME (3 mg kg^{-1}, i.v.; n=5) or phenylephrine (400 µg kg^{-1} h^{-1}, i.v.; n=5).

Data presentation and analysis

All data in the text are given as means ± s.e.mean. The values after the different treatments were compared to the baseline values by the use of Student's *t* test, once analysis of variance had revealed that the samples represented different populations. The significance of changes induced by L-NAME or phenylephrine, as compared to the respective saline treatment, was tested by the Student Newman-Keuls test for multiple comparison. In case of 5-CT and DOI, differences among groups were

compared with the Student-Newman-Keuls test for multiple comparison (5-CT) or Student's *t* test (DOI). A P value of 0.05 or less was considered statistically significant.

Drugs

The compounds used in this study were: 5-Carboxamidotryptamine maleate (gift: Glaxo Group Research, Ware, U.K.), (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-amino-propane (DOI), 5-hydroxytryptamine creatinine sulphate, N^G-nitro-L-arginine methyl ester (all from Sigma, St. Louis, U.S.A.), ketanserin tartrate (gift: Janssen Pharmaceuticals, Beerse, Belgium), phenylephrine hydrochloride (Department of Pharmacy, Erasmus University, Rotterdam, The Netherlands), sodium pentobarbitone (Sanofi BV, Maassluis, The Netherlands). All compounds were dissolved in sterile saline. The doses mentioned in the text refer to the respective salts.

3.3 Results

Haemodynamics

The blood pressure and heart rate characteristics of the three groups of pithed rats, used for the analysis of hypotensive (with ketanserin treatment) and hypertensive (without ketanserin) responses to 5-HT, are depicted in Figure 3.1. No changes in blood pressure occurred with saline, but dose-dependent hypertension was noticed after both L-NAME and phenylephrine. The increases in mean arterial blood pressure caused by L-NAME were similar in animals that had been treated with 0.1 mg kg^{-1} ketanserin (baseline, 59±2 mm Hg; maximum, 125±11 mm Hg) and those that had not (baseline, 59±1 mm Hg; maximum, 126±6 mm Hg). Also, the pressor responses to the infusions of phenylephrine did not differ in the presence (baseline, 54±2 mm Hg; maximum, 124±5 mm Hg) or absence (baseline, 50±2 mm Hg; maximum, 124±3 mm Hg) of ketanserin. While heart rate did not change after L-NAME, the higher doses of phenylephrine caused tachycardia in both ketanserin-treated and untreated rats (Figure 3.1).

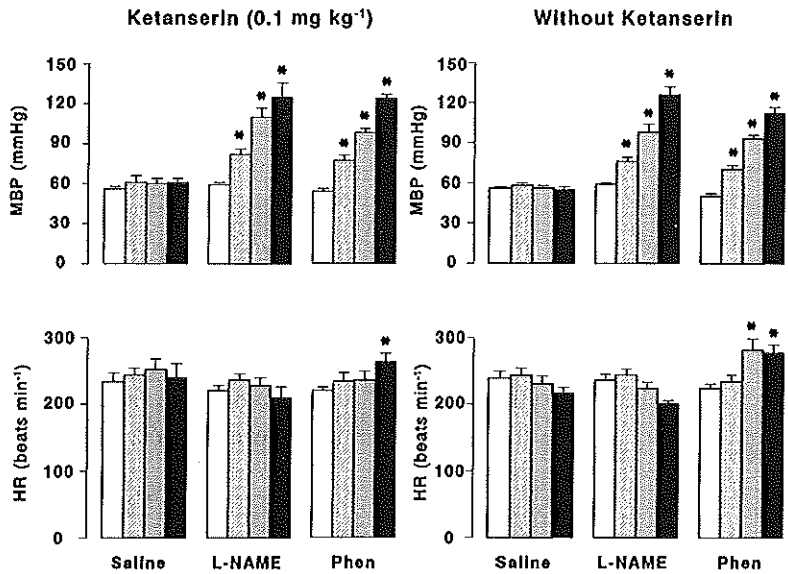

Figure 3.1 Mean arterial blood pressure (MBP) and heart rate (HR) values in pithed rats with (left panels) or without (right panels) treatment with ketanserin in groups which received either saline (n=7 and 8, respectively), L-NAME (n=13 and 15, respectively) or phenylephrine (n=9 and 8, respectively). In each set of panels, the 4 columns from left to right represent, sequentially, values before and after the three doses of saline (three times 0.5 ml kg^{-1}), L-NAME (1, 3 and 10 mg kg^{-1}) or phenylephrine (160, 240 and 400 μg kg^{-1} h^{-1}). Data are given as mean ± s.e.mean. *, $P<0.05$ versus saline.

Hypotensive responses to 5-HT

The hypotensive responses elicited by 5-HT (0.1-3 μg kg^{-1}), after treatment with ketanserin (0.1 μg kg^{-1}), in pithed rats before and after saline, L-NAME or phenylephrine are shown in Figure 3.2. These doses of 5-HT elicited a moderate hypotensive response which remained unaltered after the three doses of saline. However, in the group treated with L-NAME hypotensive responses to 5-HT (1 μg kg^{-1}; n=6-10) were significantly augmented by 3 and 10 mg kg^{-1} L-NAME. The response to 5-HT (3 μg kg^{-1}; n=6-9) was enhanced by all three doses of L-NAME (1, 3 and 10 mg kg^{-1}). At similar blood pressure levels during phenylephrine infusions, the responses to 5-HT were also augmented. The changes in hypotensive responses, compared to the

respective control responses, were similarly potentiated in both L-NAME and phenylephrine treated animals (Figure 3.2).

The duration of action of the hypotensive action of 5-HT in the three groups of animals was also studied (Figure 3.3). With the two highest doses of L-NAME the time to reach 50 % recovery of the response to 5-HT (1 μg kg^{-1}) was significantly prolonged. The duration of the responses to 5-HT (3 μg kg^{-1}) also tended to increase after L-NAME, but the changes were not significant. Phenylephrine did not affect the duration of action of 5-HT.

Hypotensive responses to 5-CT

As shown in Figure 3.4, compared to saline-treated animals the hypotensive responses to 5-CT (0.1-3.0 μg kg^{-1}) were significantly and dose-dependently potentiated in animals treated with L-NAME (1, 3 or 10 mg kg^{-1}).

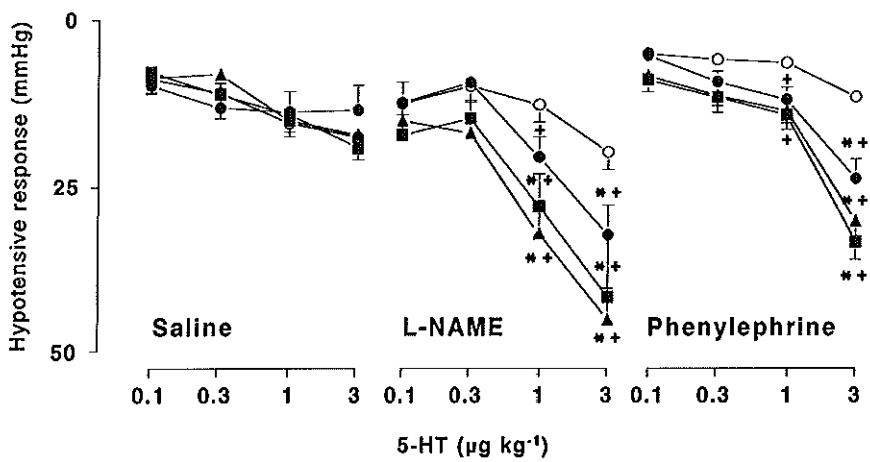

Figure 3.2 Hypotensive response to 5-HT (0.1-3.0 μg kg^{-1}) in pithed rats treated with ketanserin (0.1 mg kg^{-1}) before (○) and after treatment with saline (0.5 ml kg^{-1} three times; n=5-6), L-NAME (1, 3 and 10 mg kg^{-1}; n=6-10) or phenylephrine (160, 240 and 400 μg kg^{-1} h^{-1}; n=6-7). The three doses in each case are represented, respectively, by the symbols (●), (▲) and (■). Data are given as mean ± s.e.mean. *, $P<0.05$ versus saline; +, significant change from control response compared versus the change in saline ($P<0.05$).

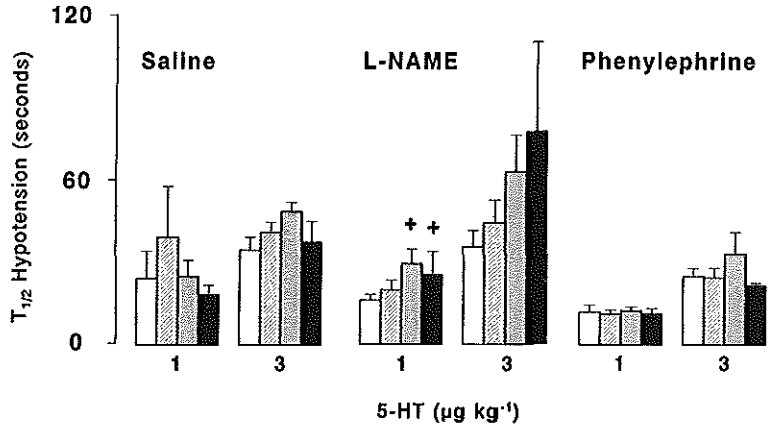

Figure 3.3 Time needed for 50% recovery ($T_{1/2}$) of the hypotensive action of 5-HT (1 and 3 µg kg^{-1}) in pithed rats treated with ketanserin (0.1 mg kg^{-1}) in groups which received either saline, L-NAME or phenylephrine. In each set of panels, the 4 columns from left to right represent, sequentially, values before (control) and after the three doses of saline (three times 0.5 ml kg^{-1}; n=5-7), L-NAME (1, 3 and 10 mg kg^{-1}; n=6-10) or phenylephrine (160, 240 and 400 µg kg^{-1} h^{-1}; n=6-7). Data are given as mean ± s.e.mean. +, P<0.05 versus control responses.

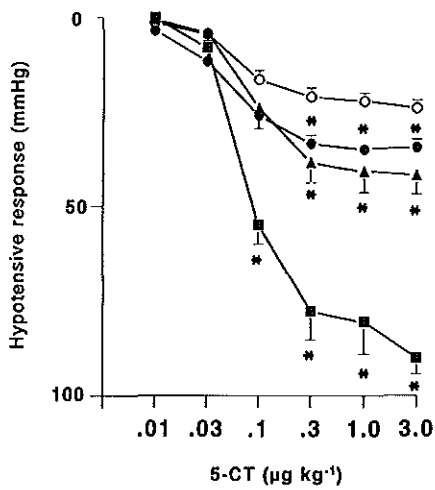

Figure 3.4 Hypotensive responses to 5-CT (0.01-3.0 µg kg^{-1}; cumulative) in pithed rats treated with either saline (control;○, n=5) or L-NAME (1, 3 and 10 mg kg^{-1}; n=5 each). The three doses are represented, respectively, by the symbols (●), (▲) and (■). Data are given as mean ± s.e.mean. *, P<0.05 versus saline.

Hypertensive responses to 5-HT

Before any treatment 5-HT (3, 10 and 30 μg kg^{-1}) induced a dose-dependent pressor response, which in the saline group reached a peak of 52±3 mm Hg and a $T_{1/2}$ of 19±1 s after the highest dose. Both L-NAME (up to 10 mg kg^{-1}) and phenylephrine (up to 400 μg kg^{-1} h^{-1}) failed to alter the magnitude of the 5-HT-induced pressor effects (Figure 3.5). In contrast to phenylephrine, L-NAME (3 mg kg^{-1}) prolonged the duration of the responses to 5-HT (30 μg kg^{-1}; n=7-9), compared to the corresponding baseline values. The highest dose of L-NAME prolonged the duration significantly, both compared to the corresponding baseline value as well as saline values (Figure 3.6).

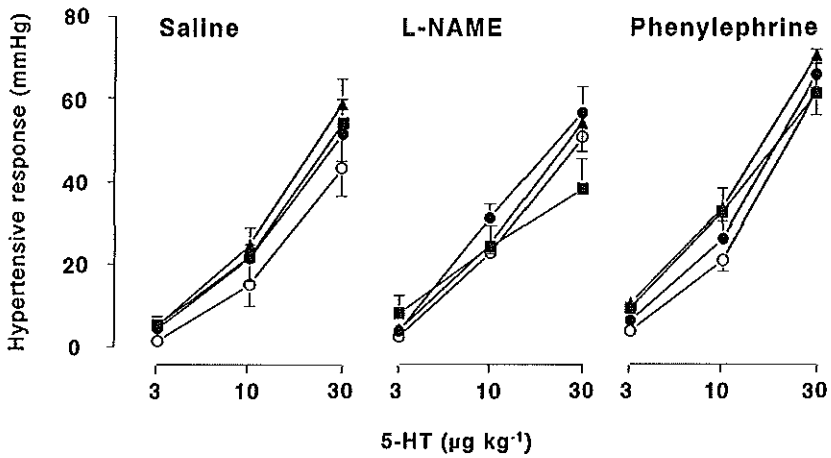

Figure 3.5 Hypertensive responses to 5-HT (3-30 μg kg^{-1}) in pithed rats before (control; ○) and after treatment with saline (0.5 ml kg^{-1} three times; n=7-8), L-NAME (1, 3 and 10 mg kg^{-1}; n=7-9) or phenylephrine (160, 240 and 400 μg kg^{-1} h^{-1}; n=6-7). The three doses in each case are represented, respectively, by the symbols (●), (▲) and (■). Data are given as mean ± s.e.mean. *, P<0.05 versus the corresponding value in the saline experiments.

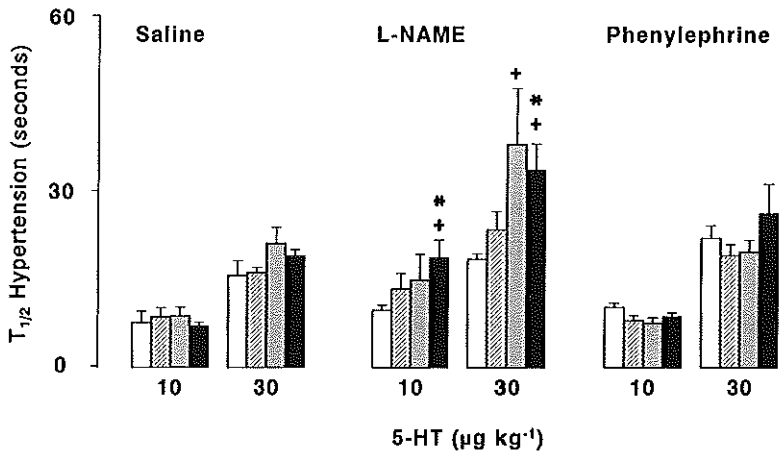

Figure 3.6 Time needed for 50% recovery ($T_{1/2}$) of the hypertensive action of 5-HT (10 and 30 µg kg^{-1}) in pithed rats in groups which received either saline, L-NAME or phenylephrine. In each set of panel, the 4 columns from left to right represent, sequentially, values before (control) and after the three doses of saline (three times 0.5 ml kg^{-1}; n=7), L-NAME (1, 3 and 10 mg kg^{-1}; n=7-9) or phenylephrine (160, 240 and 400 µg kg^{-1} h^{-1}; n=6-7). Data are given as mean ± s.e.mean. *, $P<0.05$ versus the corresponding value in the saline experiments. +, $P<0.05$ versus control responses.

Hypertensive responses to DOI

The hypertensive responses to DOI (3, 10 and 30 µg kg^{-1}) were enhanced after treatment of the animals with L-NAME (3 mg kg^{-1}), but remained essentially unchanged after the infusion of phenylephrine (400 µg kg^{-1} h^{-1}). The hypertensive effect of the two highest doses (10 and 30 µg kg^{-1}) of DOI was significantly more in animals treated with L-NAME than in animals treated with phenylephrine (Figure 3.7; upper panels). The time needed to 50% recovery of the response to DOI (10 µg kg^{-1}) was enhanced by L-NAME, whereas the duration of the response to the highest dose of DOI (30 µg kg^{-1}) was slightly reduced. In contrast, phenylephrine infusion decreased the duration of the hypertension action of DOI (10 and 30 µg kg^{-1}) (Figure 3.7; lower panels).

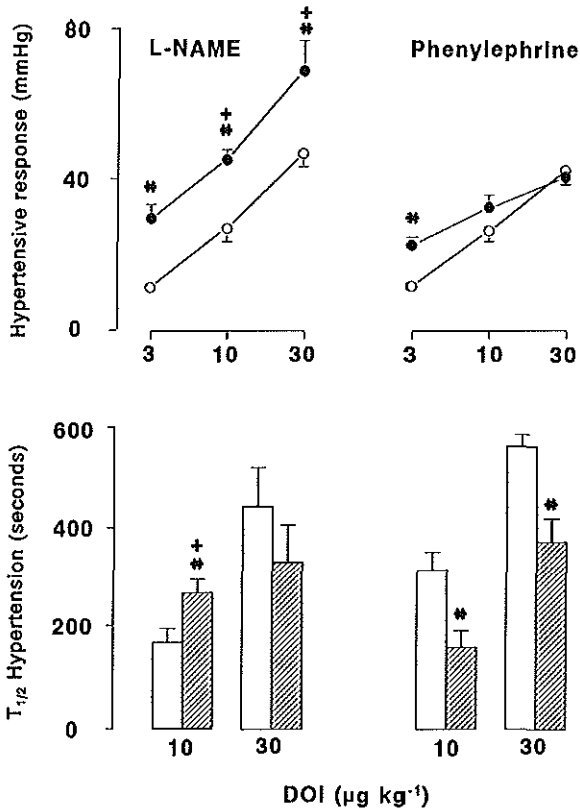

Figure 3.7 Hypertensive responses (upper panels) and time needed for 50% recovery ($T_{1/2}$) of the hypertensive responses (lower panels) induced by DOI (3-30 µg kg^{-1}) in pithed rats before (control; o or open columns) and after (● and hatched columns) treatment with L-NAME (3 mg kg^{-1}; n=5) or phenylephrine (400 µg kg^{-1} h^{-1}; n=5). Data are given as mean ± s.e.mean. *, P<0.05 versus the corresponding value in the saline experiments. +, P<0.05 versus control responses.

3.4 Discussion

Blood pressure and heart rate changes by L-NAME

There is increasing evidence in several species, including the rat, that NO maintains a basal vasodilator tone. This tone seems to be independent of the existing vascular tone as blood pressure was increased to a similar extent by L-NAME in pithed rats (present results) or anaesthetized rats (chapter 2). However, the underlying mechanism of the increase in blood pressure by NO-synthase inhibitors, such as L-NAME, is still poorly understood. In any case, a contribution of the autonomic nervous system or the renin-angiotensin system seems to be unlikely as the pressor responses to L-NNA in the rat are unaffected by blockade of the autonomic nervous system (Wang & Pang, 1991) or the angiotensin I-converting enzyme (Wang and Pang, 1991; Zambetis *et al.*, 1991). Furthermore, in a dose (0.1 mg kg^{-1}) that is sufficient to block 5-HT_2 receptors (Kalkman *et al.*, 1982), ketanserin failed to alter the increasesin blood pressure due to L-NAME in our experiments. Since the pressor responses to phenylephrine infusions were also similar (see Figure 3.1), the above dose of ketanserin apparently did not affect α_1-adrenoceptors in our experiments. Though this finding is in agreement with those of Kalkman *et al.* (1982), Fozard (1982) did observe antagonism of phenylephrine-induced hypertension with similar doses of ketanserin.

The bradycardia observed after L-NAME in both anaesthetized and conscious rats (Gardiner *et al.*,1990; Rees *et al.*, 1990; Wang & Pang, 1991; chapter 3) is likely to be largely reflex-mediated. Thus, few changes in heart rate occurred with L-NAME in the present experiments in pithed rats, as well as in intact rats after ganglion blockade (Fozard & Part, 1991) or atropine (Widdop *et al.*, 1992). Moreover, the bradycardia and decrease in renal sympathetic nerve activity induced by N^G-methyl-L-arginine (L-NMA) in the urethane anaesthetized rat were attenuated by baroreceptor deafferentation (Sakuma et al., 1992).

Hypotensive responses to 5-HT and 5-CT

It increasingly appears that there is a dissimilarity in the effects of L-NAME on the vasodilator responses to acetylcholine observed in experiments *in vitro* and *in vivo*. The vasodilator responses to acetylcholine, being susceptible to endothelial damage or NO-synthase inhibitors, almost exclusively depend on the release of NO in a number of isolated blood vessels (see Furchgott & Vanhoutte 1989), whereas this does not seem to be the case *in vivo* as the peak hypotension induced by acetylcholine were not

attenuated, though the duration was somewhat decreased by L-NAME (chapter 2). One of the reasons of this discrepancy could be that experiments *in vitro* are usually performed on relatively large (conduit) vessels, whereas the outcome *in vivo* is mainly determined by small (resistance) vessels, i.e. arterioles.

In the present study, hypotensive responses induced by low doses of 5-HT in the presence of ketanserin were not attenuated, but were even significantly enhanced, by L-NAME. Since the enhancement of the hypotensive response to 5-HT was also seen in animals treated with phenylephrine, it appears that the potentiation of the 5-HT-induced hypotension is most likely due to an increase in blood pressure rather than to interference with the L-arginine-NO pathway. This finding is in contrast to the results obtained with 5-HT in the perfused coronary (Mankad *et al.*, 1991), or renal (Verbeuren *et al.*, 1991) arteries of the rat or with α-methyl-5-HT in the rabbit isolated jugular vein (Martin et al, 1992). The potentiating effect of L-NAME in our experiments was even more pronounced in experiments in which 5-CT was used. This 5-HT_1 receptor agonist has been shown to induce endothelium-dependent as well as independent relaxant responses, depending on the vessel under study (Schoeffter & Hoyer, 1990; Sumner, 1991; Verbeuren *et al.*, 1991). Hence, it is not possible to exclude fully the release of NO *in vivo*, but it is likely that the hypotensive response to 5-HT and 5-CT is largely mediated by a direct vasodilator action on smooth muscle cells.

Hypertensive responses to 5-HT and DOI

Inhibition of the basal NO-release did not change the magnitude of the 5-HT-induced pressor responses, but the duration of the effects was moderately prolonged. Therefore, it would appear that the release of NO seems to limit the hypertensive effects of 5-HT to some extent. The involvement of a NO-specific mechanism is indicated by the finding that phenylephrine failed to alter either the magnitude or the duration of the 5-HT effect. Indeed, the presence of intact endothelium has been shown to counteract contractions in response to 5-HT in the rat isolated mesenteric and coronary arteries (Berg-Nyborg & Mikkelsen, 1990; Dohi & Lüscher, 1991), and treatment with N^G-monomethyl-L-arginine (L-NMMA) converts 5-HT-induced relaxations into contractions in the rat coronary artery (Mankad et al, 1991). Whether the contractions are opposed by basal or receptor-stimulated NO-release is not known. However, in the dog perfused coronary arteries contractions due to 5-HT are similarly augmented by L-NNA and L-NMMA, whereas contractions

to prostaglandin $F_{1\alpha}$ remain unaffected (Cappelli-Bigazzi *et al.*,1991). Likewise, L-NAME enhances 5-CT and sumatriptan contractions in the dog perfused kidneys, but fails to alter those to angiotensin II (Whiting & Cambridge, 1991). Moreover, endothelial denudation augments contractions induced by 5-HT as well as several 5-HT_1 receptor agonists in the dog isolated basilar artery (Connor & Feniuk, 1989). These findings are indicative of a specific receptor mediated NO-release.

So far, no endothelium-dependent response has been reported for the 5-$HT_{1C/2}$ receptor agonist DOI. We now demonstrate that the magnitude of DOI responses was augmented by the NO-synthase inhibitor L-NAME. These responses were much more potentiated than the comparable hypertensive responses to 5-HT. Furthermore, the duration of the response to the middle dose of DOI was prolonged. Pressor effects elicited by 5-HT could be opposed by three possible vasodilator actions involving a direct arteriolar dilatation (see above) or a 5-HT_1 receptor-mediated or 'contraction-induced' NO-release from the endothelium. In contrast, the responses to DOI may be solely opposed by the endothelial release of NO and, thus, become more pronounced than those to 5-HT after L-NAME. Whether NO-release is specifically related to 5-HT_2-receptor mediated pressor responses *in vivo* or a more general phenomenon related to pressor responses *per se*, is still not clear. Pressor responses induced by phenylephrine are slightly potentiated by L-NAME in ganglion-blocked rats (Chyu et al., 1992), but are unaffected by L-NNA in conscious rabbits (Du et al., 1992). Furthermore, responses to noradrenaline in pentobarbitone-anaesthetized rats are unaffected after L-NMMA (Gray et al., 1991). Finally, one should bear in mind that the pressor responses to DOI, at least in conscious rats, are in part mediated renin release (Alper, 1990).

In conclusion, L-NAME increases arterial blood pressure in animals with a low vascular tone. 5-HT- and 5-CT-induced hypotensive responses seem to be largely independent of NO release by the endothelium. However, the hypertensive responses to 5-HT and DOI appear to be limited by the release of NO in the pithed rat.

Chapter 4

N^G-Nitro-L-arginine methyl ester: systemic and pulmonary haemodynamics, tissue blood flow and arteriovenous shunting in the pig

Summary

The systemic and pulmonary haemodynamic effects of N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of the endothelial nitric oxide (NO) biosynthesis, were evaluated in the anaesthetized pig. Coincidently, local tissue and arteriovenous anastomotic blood flows were investigated at baseline and following L-NAME, using simultaneous injections of radioactive microspheres of two different sizes (diameter: 15 and 50 μm). L-NAME (1, 3 and 10 mg kg^{-1}) reduced systemic and pulmonary artery conductance and cardiac output, but heart rate and mean arterial blood pressure remained unchanged. L-arginine (1000 mg kg^{-1}) reversed the systemic and pulmonary haemodynamic changes induced by L-NAME.

As detected with 15 μm microspheres, L-NAME (1 and 3 mg kg^{-1}) decreased tissue blood flow to and vascular conductance in the eyes, lungs, atria, kidneys, adrenals and liver. Furthermore, the difference between blood flows simultaneously measured with 15 and 50 μm microspheres, which can be equated to blood flow through arteriovenous anastomoses with a diameter between about 28 and 90 μm, was reduced by L-NAME (3 mg kg^{-1}) in the skin of head and gluteal regions and, as indicated by the microsphere content of the lungs, in the total systemic circulation.

These results suggest that in the anaesthetized pig (i) NO is involved in the regulation of both systemic and pulmonary vascular conductance, (ii) the decrease in systemic vascular conductance is in part due to constriction of systemic arteriovenous anastomoses, and (iii) the decrease in pulmonary vascular conductance, leading to reduction of cardiac output, seems to negate the expected rise in arterial blood pressure observed, for example, in rats and rabbits following inhibition of NO-synthesis.

Based on: Van Gelderen E.M., Den Boer M.O. and Saxena P.R. (1993). N^G-nitro-L-arginine methyl ester: systemic and pulmonary haemodynamics, tissue blood flow and arteriovenous shunting in the pig. Naunyn-Schmiedeberg's Arch. Pharmacol. 348; 417-423.

4.1 Introduction

Vascular smooth muscle tone is regulated by the release of endothelium-derived relaxing factor (EDRF), which has now been identified as nitric oxide (NO) (Ignarro *et al.*, 1987; Palmer *et al.*, 1987) or a related nitrosothiol (Myers *et al.*, 1990). Its synthesis from the precursor L-arginine is mediated by the enzyme NOS present in endothelial cells (Palmer *et al.*, 1988; Förstermann *et al.*, 1991). Analogues of L-arginine, including N^G-nitro-L-arginine (L-NNA) and its methyl ester (L-NAME), have been shown to inhibit NO biosynthesis (Mülsch & Busse 1990; Rees *et al.*, 1990). These NOS-inhibitors induce a sustained rise in arterial blood pressure in rats (Gardiner *et al.*, 1990; Rees *et al.*, 1990; this thesis chapter 2 and 3) and rabbits (Humphries *et al.*, 1991; Du *et al.*, 1992), indicating a basal NO release mediating vasodilatation. In these species, blood pressure elevation is usually accompanied by a reflex bradycardia. Less pronounced effects on blood pressure as well as heart rate were found with NOS-inhibitors in lambs (Fineman *et al.*, 1991), goats (Garcia *et al.*, 1992), dogs (Richard *et al.*, 1991; Klabunde *et al.*, 1991) and cats (this thesis, chapter 2; McMahon *et al.*, 1992); in cats, microsphere content of the lungs, used as an *index* for systemic arteriovenous shunting, was decreased (this thesis, chapter 2). The reason for the poor pressor response following inhibition of NO synthesis in these animal species is not known, but it is possible that the pressor response due to rise in systemic vascular resistance is offset by an additional decrease in cardiac output as a consequence of a more marked increase in pulmonary vascular resistance. Indeed, NOS-inhibitors have been shown to block pulmonary vasodilator response to vagal stimulation in cats (McMahon *et al.*, 1992), attenuate endothelium-dependent pulmonary vasodilatation, leading to pulmonary hypertension, in lambs (Fineman *et al.*, 1991), and to produce pulmonary hypertension and reduced cardiac output in dogs (Klabunde *et al.*, 1991). The purpose of this investigation was to evaluate the effects of L-NAME on systemic and pulmonary haemodynamics, tissue blood flows and total and regional arteriovenous shunting, using radioactive microsphere of two different diameters (15 and 50 μm) in anaesthetized pigs. Preliminary findings have been reported elsewhere (Van Gelderen *et al.*, 1992).

4.2 Methods

General

Twelve male and female pigs (weight 17.8±0.3 kg) were anaesthetized with metomidate (120 mg, i.v.) following sedation with azaperone (150 mg, i.m.). After intubation, the animals were connected to a respirator (Bear 2E, BeMeds AG, Baar, Switzerland) for ventilation with a mixture of oxygen and room air. Arterial blood gases were measured (ABL-2, Radiometer, Copenhagen, Denmark) and kept within a physiological range (pH 7.45-7.55, PO_2 90-120 mmHg, PCO_2 35-45 mmHg) by adjusting the ventilation rate and the oxygen-content of the mixture. Anaesthesia was maintained by infusing pentobarbitone sodium in the right femoral vein; 20 mg kg^{-1} h^{-1} during the first hour followed by 12 mg kg^{-1} h^{-1}. A catheter was introduced in the left femoral artery for blood pressure measurement. Cardiac output was measured with the help of a 6F-Swan-Ganz thermodilution catheter (Corodyn, Braun Melsungen AG, Melsungen, Germany), which was advanced into the pulmonary artery via the left femoral vein and connected to a cardiac output computer (WTI, Rotterdam, The Netherlands). Another catheter was placed in the left ventricle via the left common carotid artery to inject radioactive microspheres. To calibrate the radioactive microsphere method, arterial reference samples were collected from the aorta via a catheter in the right femoral artery. Blood pressures were continuously measured in the aorta, the pulmonary artery and the left ventricle, using pressure transducers (Statham P23, Hato Rey, Puerto Rico). Mean arterial blood pressure was calculated as the sum of the diastolic pressure and one third of the difference between systolic and diastolic pressure. Heart rate, left ventricular end diastolic pressure and the peak rate of rise in left ventricular pressure (LV dP/dt) were derived from the ventricular pulse pressure signal. Pulmonary capillary wedge pressure was measured by inflating the Swan-Ganz-balloon and pulmonary artery resistance was calculated by dividing the difference between mean pulmonary artery and capillary wedge pressures by cardiac output. In some experiments the last measurement (after L-arginine) of pulmonary capillary wedge pressure could not be measured and, therefore, pulmonary vascular resistance was not calculated. Drugs were injected into the aorta to prevent high concentrations of drugs reaching the heart. All catheters were filled with a heparin sodium solution (80 IU ml^{-1}) to prevent clotting. Body temperature was kept above 37°C and physiological saline was infused to compensate for fluid loss. The animals were allowed to stabilize for at least 45 min before the experiment started.

Radioactive microsphere technique

The regional distribution of the cardiac output was determined with the radioactive microsphere technique using the reference blood smple method (Heymann *et al.*, 1977), modified for use of simultaneous injection of microspheres of different diameter (see Saxena & Verdouw 1985; Den Boer *et al.*, 1992a,b). The microspheres used in this investigation were of 15 and 50 μm diameter and labelled with either ^{141}Ce (15 μm), ^{113}Sn (50 μm), ^{103}Ru (50 μm), ^{95}Nb (15 μm) or ^{46}Sc (15 μm) (NEN Company, Dreieich, Germany). The order of 15 and 50 μm spheres injected before and after the drugs was randomized. The approximate number of microspheres given per isotope was about 1,000,000 for 15 μm and 50,000 for 50 μm. The microspheres were vortexed for about 30 s and then injected into the left ventricle against the direction of the blood flow to ensure uniform mixing. An arterial reference sample was withdrawn (rate: 10.5 ml min^{-1}) using a pump (Braun-Melsungen, Melsungen, Germany), starting about 15 s before and continuing until 1 min after the injection of the microspheres. At the end of the experiment the animals were killed by an overdose of sodium pentobarbitone and the different tissues were dissected out, weighed and put in vials. The radioactivity in these vials and in the blood samples was counted for 5-10 min in a gamma-scintillation counter (Packard, Minaxi Autogamma 5000) using suitable windows for discriminating the different isotopes. For each tissue, blood flow (Q_{tis}) was calculated using the formula: $Q_{tis} = (I_{tis} / I_{ref}) \times 10.5$, in which I_{tis} and I_{ref} represent, respectively, radioactivity in the tissue and in the reference blood sample. Tissue vascular conductance was calculated by dividing the tissue blood flow value by mean arterial blood pressure. Tissue blood flow and conductance values have been expressed per 100 g of tissue. All data were processed by a personal computer (Olivetti PCS 286) using a set of specially developed computer programs, based on those described earlier (Saxena *et al.*, 1980).

As extensively discussed elsewhere (Johnston & Saxena 1978; Saxena & Verdouw 1985; Den Boer *et al.*, 1992a,b), not all microspheres are trapped in tissues; they escape entrapment via arteriovenous anastomoses to appear into the venous blood and are ultimately sieved in the lungs. Lung radioactivity, therefore, represents for the greatest part arteriovenous anastomotic blood flow, although a small part (1-1.5% of cardiac output) is derived from the bronchial arteries (Baille *et al.*, 1982; Wu *et al.*, 1988). Based on the demonstration in the hamster cheek pouch that microspheres of 15 and 24 μm diameter are already trapped in blood vessels with a diameter of 27.7 and 42.7 μm, respectively (Dickhoner *et al.*, 1978), it may be assumed that 15 μm

spheres detected in a certain tissue reflect blood flow through vessels with a diameter smaller than about 28 μm (capillaries and small arteriovenous anastomoses), whereas 50 μm spheres reflect blood flow through vessels under 90 μm diameter (capillaries and medium-sized arteriovenous anastomoses). Therefore, the difference between blood flow values simultaneously obtained with 15 and 50 μm microspheres may serve as an *index* of the blood flow in arteriovenous anastomoses with a diameter between 28 and 90 μm.

Experimental protocol

At baseline values of systemic and pulmonary haemodynamic variables were collated and a mixture of 15 and 50 μm microspheres was injected in the left ventricle to determine baseline regional tissue blood flows. The animals were then divided at random into two groups (n=6 each), which were treated every 25 min with three consecutive doses of L-NAME (1, 3 and 10 mg kg^{-1}) or saline (3 ml each). Systemic and pulmonary haemodynamic values were collected after each dose of L-NAME or saline. After the first dose 15 μm microspheres were injected, whereas after the second dose 15 and 50 μm spheres were injected simultaneously. Finally, in both groups five animals were treated with L-arginine (1000 mg kg^{-1}) and, 15 min later, the haemodynamic variables, except pulmonary capillary wedge pressure, were collated again.

Data presentation and analysis

All data in the text are mentioned as mean ± s.e.mean. The effects of treatments were analyzed in each group using a repeated measurements analysis of variance with the factors size and treatment. Once the samples represented different populations, the values after the different treatments were compared to baseline values by use of Duncan's new multiple range test. In each group the difference in blood flow values obtained with 15 and 50 μm microspheres before and after treatment were analyzed with a Student's *t* test for paired data. The changes among the two groups were tested by a Student's *t* test. Statistical significance was accepted at $P < 0.05$ (two-tailed).

Chemicals

The drugs used in this study were: azaperone, metomidate (both from Janssen Pharmaceutica, Beerse, Belgium), L-arginine, N^G-nitro-L-arginine methyl ester (both from Sigma, St. Louis, U.S.A.), pentobarbitone sodium (Narcovet, Apharmo, Arnhem,

The Netherlands) and heparin sodium (Thromboliquine, Organon Teknika B.V., Boxtel, The Netherlands). All doses refer to the respective salts.

4.3 Results

Systemic and pulmonary haemodynamics

Systemic and pulmonary haemodynamic variables in the two groups at baseline and following treatment with L-NAME or saline are depicted in Figure 4.1. Apart from mean arterial blood pressure, which was somewhat higher in the saline group, no differences were observed initially between the groups. L-NAME significantly reduced cardiac output (by 40±2 and 45±4% after 3 and 10 mg kg^{-1}, respectively) and stroke volume (by 28±3, 46±5 and 53±5% after the three doses, respectively), but did not significantly affect heart rate, mean arterial blood pressure, LV dP/dt or pulmonary capillary wedge pressure (both not shown in Figure 4.1). Left ventricular end diastolic pressure decreased significantly after 3 mg kg^{-1} L-NAME (-17±8%), whereas mean pulmonary artery pressure increased significantly after the two highest doses (51±11 and 62±11%, respectively). L-NAME significantly reduced systemic (by 30±3, 36±6 and 28±11% after the three doses, respectively) and pulmonary (by 65±5 and 65±2% after 3 and 10 mg kg^{-1}, respectively) vascular conductances (Figure 4.1).

Except for arterial blood pressure, administration L-arginine (1000 mg kg^{-1}) partially or fully reversed the systemic and pulmonary haemodynamic changes induced by L-NAME.

Tissue blood flow and vascular conductance

Tissue blood flows measured with 15 μm radioactive microspheres at baseline and after L-NAME are shown in Figure 4.2. L-NAME, 1 and 3 mg kg^{-1}, respectively, decreased blood flow to the eyes (42±5 and 62±4%), atria (37±8 and 44±9%), lungs (42±8 and 68±5%), kidneys (31±2 and 51±4%), and adrenals (35±7 and 60±7%). When compared to baseline values, blood flow was reduced in the bones (32±5 and 39±6%) and liver (32±12%, 1 mg kg^{-1} only). L-NAME induced no significant change in blood flow to the brain as well as to the skin, tongue, spleen, stomach, mesentery, intestines or skeletal muscles (not shown in Figure 4.2).

Since mean arterial blood pressure changed only moderately during the course of the experiment, similar changes were observed in tissue vascular conductances.

The vascular conductance was decreased in the adrenals (37±7 and 59±5%), eyes (45±5 and 60±4%), kidneys (35±4 and 48±4%), bones (36±5 and 35±8%) and lungs (46±7 and 67±5%) by L-NAME (1 and 3 mg kg^{-1}, respectively). Additionally, the lower dose of L-NAME also reduced vascular conductance in the liver (35±12%) and heart (16±5%). In the heart, the effect of L-NAME was not observed in the ventricles and was confined to the atria, where vascular conductance was decreased by 42±6 and 44±6% by 1 and 3 mg kg^{-1} of L-NAME, respectively.

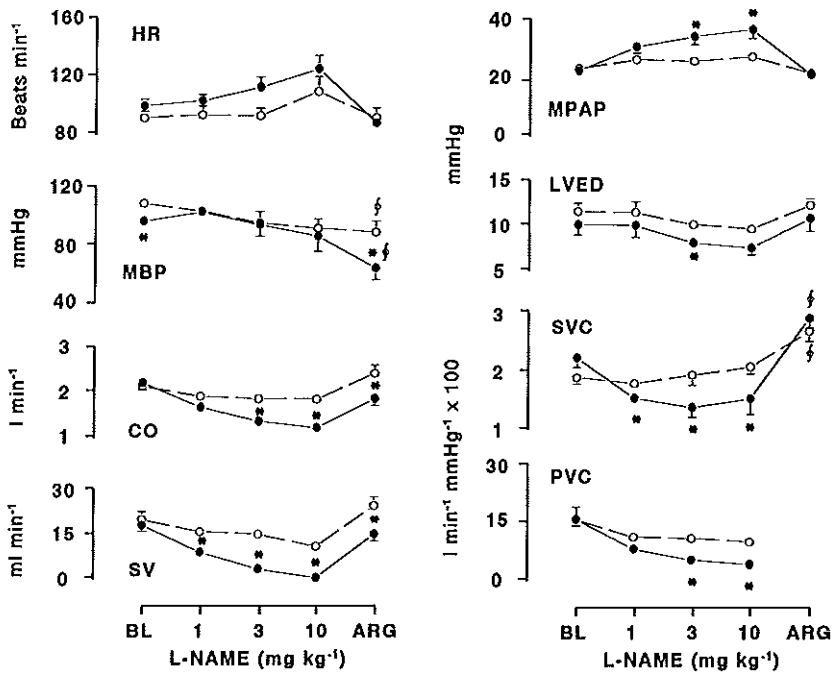

Figure 4.1 Systemic haemodynamics in anaesthetized pigs before (BL) and after saline (open circles) or L-NAME (closed circles), followed in both groups (n=6 each) by L-arginine (1000 mg kg^{-1}; ARG). The haemodynamic variables are abbreviated: heart rate (HR), mean arterial blood pressure (MBP), cardiac output (CO), stroke volume (SV), mean pulmonary artery pressure (MPAP), left ventricular end diastolic pressure (LVEDP), systemic vascular conductance (SVC) and pulmonary artery conductance (PVC). Data are given as mean±s.e. mean, but in some cases error bars fall within the limits of the symbol. *, $p<0.05$ versus corresponding saline value; §, $p<0.05$ versus baseline value, in case of ARG only.

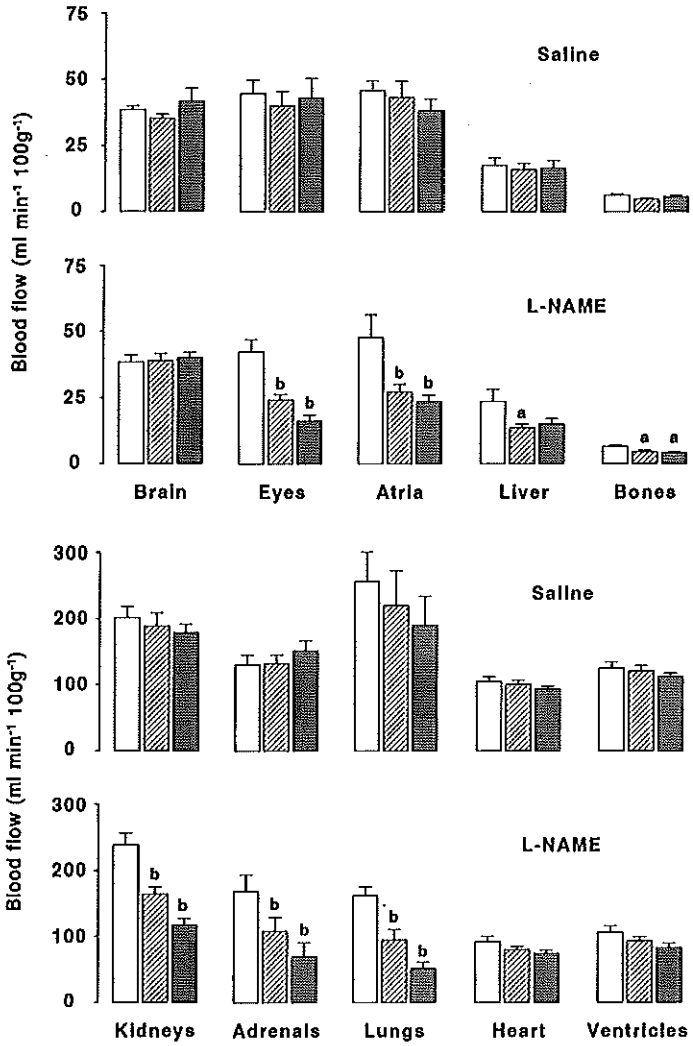

Figure 4.2 Tissue blood flows measured with 15 μm radioactive microspheres in pigs treated with either saline (upper panels; n=6) or L-NAME (lower panels; n=6). In each set of panels, baseline blood flow is shown by the open bars, whereas increasing doses of L-NAME (1 and 3 mg kg^{-1}) or saline are represented by the hatched and cross hatched bars, respectively. Data are given as mean±s.e. mean. a, $p<0.05$ versus baseline only; b, $p<0.05$ versus saline experiments.

Total systemic and regional arteriovenous anastomotic blood flow

The presence of a substantial number of 15 and 50 μm microspheres, with the latter in preponderance, in the lungs demonstrated that abundant systemic arteriovenous shunting occurs in anaesthetized pigs (Table 4.1; Figure 4.3). L-NAME (3 mg kg^{-1}) clearly reduced the number of both 15 and 50 μm microspheres as well as the difference between these two spheres (62±6%), indicating a decrease in total systemic shunting through arteriovenous anastomoses between 28 and 90 μm diameter (Figure 4.3). At the tissue level, similar blood flow values were found with the two sizes of microspheres in all tissues dissected, except in the skin and tongue (Table 4.1). The difference in the blood flows measured with 15 and 50 μm microspheres in the skin and tongue reflects the presence of arteriovenous anastomoses in these tissues. Administration of L-NAME (3 mg kg^{-1}) significantly reduced this difference in the skin (by 75±5%), but not in the tongue (Figure 4.3).

It may be noted that skin obtained from different regions showed heterogeneity (Figure 4.4). Thus, the head and gluteal skin exhibited a large difference between 15 and 50 μm microsphere blood flow values, while those from the abdominal and back

Table 4.1 Tissue blood flows (ml min^{-1} 100 g^{-1}) at baseline measured with 15 μm and 50 μm radioactive microspheres, as well as their difference (n=12).

Tissue	Microsphere size		Difference
	15 μm	50 μm	50 μm - 15 μm
Lungs§	210 ± 29	90 ± 15*	-120 ± 17
Brain	39 ± 1.5	41 ± 2.1	2.8 ± 1.0
Eyes	44 ± 3.4	48 ± 3.1	4.6 ± 2.0
Tongue	7 ± 0.4	9 ± 0.6*	1.8 ± 0.4
Bones	6 ± 0.4	7 ± 0.6	0.6 ± 0.5
Heart	99 ± 5.6	104 ± 4.5	5.1 ± 1.7
Liver	21 ± 2.8	22 ± 3.2	1.6 ± 0.6
Spleen	111 ± 15	119 ± 17	8.1 ± 3.3
Stomach	18 ± 1.6	19 ± 1.8	1.0 ± 0.7
Mesentery	3 ± 0.4	6 ± 1.4	2.5 ± 1.3
Intestines	39 ± 1.3	40 ± 1.5	1.0 ± 0.8
Kidneys	220 ± 13	232 ± 14	12 ± 4.5
Adrenals	155 ± 15	153 ± 20	-2.2 ± 7.2
Skin	5 ± 0.3	21 ± 2.4*	16 ± 2.6
Muscles	5 ± 0.4	5 ± 0.4	0.4 ± 0.2

§, indicates the sum of total systemic arteriovenous anastomotic and bronchial blood flows; *, $P<0.05$ versus the corresponding 15 μm value.

region did not. The blood flow differences were significantly decreased by L-NAME (3 mg kg^{-1}) in the gluteal and head skin by 70±5% and 70±4%, respectively (Figure 4.4).

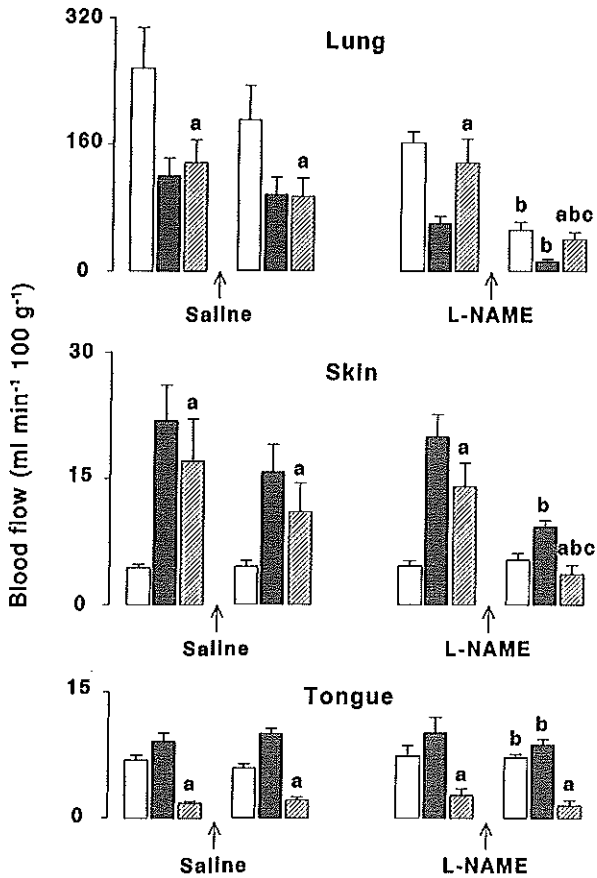

Figure 4.3 The effect of saline (n=6) or L-NAME (3 mg kg^{-1}; n=6) on total systemic (lung) and regional arteriovenous anastomoses blood flow, represented by the difference in blood flow measured with 15 μm (open bars) and 50 μm (cross-hatched bars) radioactive microspheres. In each set of panels the absolute difference is depicted by the hatched bar. Data are given as mean ± s.e. mean. a, denotes significant difference between 15 μm and 50 μm ($p<0.05$); b, denotes significant difference from baseline ($p<0.05$); c, denotes significant change in difference between 15 and 50 μm ($p<0.05$).

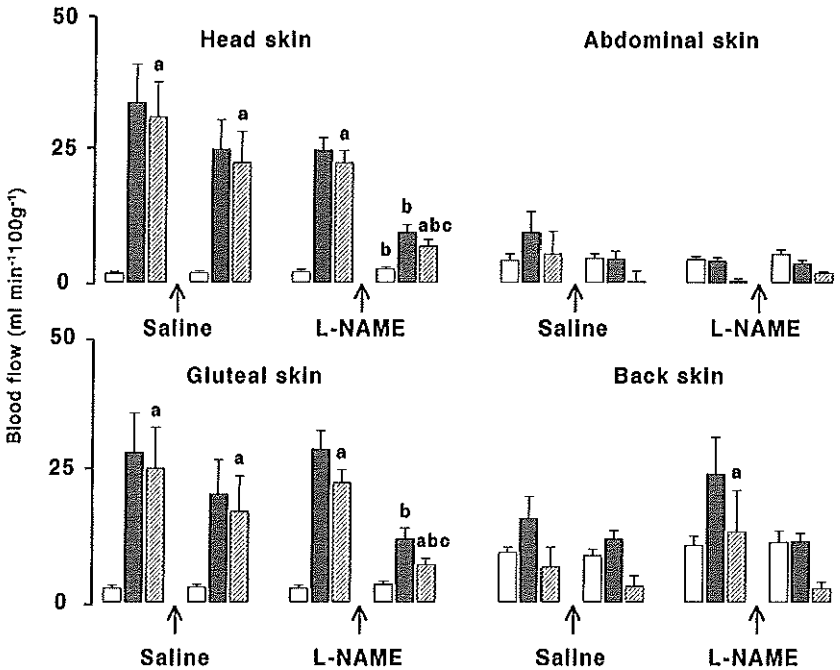

Figure 4.4 The effect of saline (n=6) or L-NAME (3 mg kg^{-1}; n=6) on arteriovenous anastomoses blood flow in several skin regions, represented by the difference in blood flow measured with 15 μm (open bars) and 50 μm (cross-hatched bars) radioactive microspheres. In each set of panels the absolute difference is depicted by the hatched bar. Data are given as mean±s.e. mean. a, denotes significant difference between 15 and 50 μm ($p<0.05$); b, denotes significant difference from baseline ($p<0.05$); c, denotes significant change in difference between 15 and 50 μm ($p<0.05$).

4.4 Discussion

The present investigation was devoted to study the effects of NO-synthase inhibition in anaesthetized pigs using L-NAME. Recently, Buxton and coworkers reported that several alkyl esters of L-arginine analogues, including L-NAME, have atropine-like properties in both radioligand binding and functional *in vitro* studies.

However, muscarinic cholinergic blockade is not likely to play a meaningful role in our studies. Firstly, *in vivo* L-NAME is rapidly dealkylated into L-NNA, which seems to be responsible for the pharmacological actions in intact animals (Schwarzacher & Raberger, 1992), but is devoid of antimuscarinic property (Buxton *et al.*, 1993). Secondly, in our previous studies in both rats and cats, L-NAME (up to 30 mg kg^{-1}) failed to attenuate the magnitude of hypotensive responses to acetylcholine (chapter 2). Lastly, the effects of L-NAME in the present study were partly reversed by L-arginine, which does not affect the antimuscarinic action of L-NAME (Buxton *et al.*, 1993).

Systemic and pulmonary haemodynamics

Several studies have demonstrated a pronounced increase in blood pressure following administration of L-NNA or L-NAME to rats (Gardiner *et al.*, 1990; Rees *et al.*, 1990; this thesis Chapter 2 and 3) and rabbits (Wiklund *et al.*, 1990); this effect is thought to be due to vasoconstriction resulting from attenuation of basal NO-release. In contrast, the pressor responses are often quite moderate or even absent in dogs, goats, lambs and cats (Fineman *et al.*, 1991; Klabunde *et al.*, 1991; Garcia *et al.*, 1992; this thesis Chapter 2). Similar to our previous observations in cats (This thesis, chapter 2), the present experiments show that L-NAME did not increase blood pressure in anaesthetized pigs, though systemic vascular conductance was significantly decreased. Coincident with the reduction in systemic vascular conductance, pulmonary vascular conductance, left ventricular end diastolic pressure, stroke volume and, ultimately, cardiac output declined dose-dependently. It is therefore likely that the expected rise in arterial blood pressure due to systemic vasoconstriction was negated by the fall in cardiac output in the pig. A comparable fall in cardiac output has also been observed in the anaesthetized dog after the NOS-inhibitors, L-NNA and N^G-monomethyl-L-arginine (Klabunde *et al.*, 1991; Perrella *et al.*, 1992).

The reduction of stroke volume and cardiac output is best explained by the marked decrease in pulmonary vascular conductance, leading to high pulmonary artery pressure, though the reduction in systemic vascular conductance may have partly contributed. Indeed, NO-release can be involved in the maintenance of a low vascular tone within the pulmonary circulation (Wiklund *et al.*, 1990; Fineman *et al.*, 1991; Perrella *et al.*, 1991) and exogenous NO may reverse pulmonary hypertension in man (Pepke-Zaba *et al.*, 1991). The rise in mean pulmonary artery pressure following L-NAME and the ability of L-arginine to reverse the changes induced by L-NAME in our experiments are in keeping with this view.

In conscious rats a direct negative inotropic action of L-NAME has been proposed, based upon the reduction in stroke volume, LV dP/dt_{max} and peak aorta blood flow (Gardiner *et al.*, 1990). Though N^G-methyl-L-arginine (L-NMA) decreased contractility and cAMP and cGMP levels in isoprenaline-stimulated rat perfused hearts, the compound failed to reduce LV dP/dt_{max}, despite a decrease in myocardial cGMP content in unstimulated hearts (Klabunde *et al.*, 1992). In anaesthetized dogs, LV dP/dt_{max} was only slightly decreased by L-NMA (Klabunde *et al.*, 1991), and intracoronary application of L-NAME did not affect myocardial tissue perfusion measured with radioactive microspheres, though L-NAME did induce a local coronary constriction (Richard *et al.*, 1991). Similarly, in the present experiments in pigs, no evidence for a major negative inotropic effect of L-NAME was uncovered as the compound did not lower LV dP/dt or ventricular vascular conductance significantly. Therefore, a reduction in myocardial oxygen supply due to coronary artery constriction by L-NAME is also unlikely.

Tissue blood flow and vascular conductance

In the anaesthetized pig, L-NAME reduced tissue blood flow and vascular conductance in a limited number of tissues, being most apparent in the adrenals, eyes, kidneys and lungs. These findings differ from our previous results in cats in which a higher dose (30 mg kg^{-1}) of L-NAME reduced blood flows to abdominal tissues without affecting renal blood flow (This thesis, chapter 3). Furthermore, as measured by the radioactive microsphere technique, reduced blood flow was observed in most of the tissues in rats following the NOS-inhibitor N^G-monomethyl-L-arginine (Greenblatt *et al.*, 1993). These differences may be related to differences in basal NO-release between species and tissues or to differences in sensitivity of the vascular smooth muscle cells. Thus, isolated porcine renal and coronary arteries show a similar EDRF release but display a difference in sensitivity to the released EDRF (Christie & Lewis 1991). In any case, the reduced renal conductance following administration of L-NAME, an inhibitor of NO-synthase, is in accordance with studies in rats (Gardiner *et al.*, 1990; Greenblatt *et al.*, 1993), rabbits (Humphries *et al.*, 1991) and dogs (Perrella *et al.*, 1992), and are indicative of a regulatory role for NO in the renal circulation.

Total systemic and regional arteriovenous shunting

The use of radioactive microspheres to measure total systemic (Johnston & Saxena 1978; Saxena *et al.*, 1980; Saxena & Verdouw 1982) and regional (Saxena & Verdouw 1985; Den Boer *et al.*, 1992a,b) arteriovenous anastomotic blood flows has been discussed previously. Briefly, microspheres (10 μm or larger in diameter) escaping entrapment in peripheral tissues and sieved by the lung capillaries can be used as an *index* of total systemic arteriovenous shunting, whereas the difference between tissue blood flows simultaneously measured with microspheres of different diameters indicates blood flow through arteriovenous anastomoses at the tissue level. In our case, the difference between blood flows measured with 15 and 50 μm spheres represents blood flow through arteriovenous anastomoses between approximately 28 and 90 μm diameter, as suggested by the relative sizes of arterial microvessels and the therein trapped microspheres (Dickhoner *et al.*, 1978).

In the present experiments, 21% of the 15 μm microspheres injected into the heart were detected in the lungs. Since bronchial blood flow has been shown to be only 1 to 1.5% of the cardiac output (Baille *et al.*, 1982; Wu *et al.*, 1988), more than 90% of the microspheres sieved by the lungs had passed through arteriovenous anastomoses. As indicated by the difference between tissue blood flows measured simultaneously with 15 and 50 μm spheres, lungs seem to completely sieve microspheres escaping entrapment mainly in the skin, but also in the tongue and, possibly, in some other tissues which we did not dissect. Interestingly, skin exhibited regional heterogeneity, with the head and gluteal skin having a much higher arteriovenous shunting than the skin from the abdominal or back region. These results are in agreement with our previous observations (Saxena & Verdouw 1985; Den Boer *et al.*, 1992b). L-NAME substantially decreased the total systemic arteriovenous shunting as judged by the decrease in the 'lung blood flow' measured with 15 μm and 50 μm microspheres as well as the difference between 'lung blood flow' measured with the microspheres of the two sizes. At the tissue level, L-NAME significantly decreased the difference between 15 and 50 μm sphere blood flows and, therefore, shunting in medium-sized arteriovenous anastomoses in the head and gluteal skin; the apparent lack of effect in abdominal and back skin and tongue may be due to a less pronounced shunting in these tissues.

The effectiveness of L-NAME tempts us to suggest that the release of NO from endothelium regulates the tone of arteriovenous anastomoses demonstrated in porcine skin. Cutaneous arteriovenous anastomoses are supposed to have a role in temperature

and, perhaps, also in blood pressure regulation (Hales & Molyneux 1988); NO-release may provide a rapid mechanism to adapt to temperature changes. However, at this moment we cannot fully exclude that blood flow through arteriovenous anastomoses following L-NAME was reduced as a result of the fall in cardiac output, causing a diminished blood supply to these vessels.

In conclusion, our results demonstrate that basal NO release regulates both systemic and pulmonary conductance in the anaesthetized pig. The expected increase in blood pressure due to NO-synthase inhibition is offset by the coincident reduction in cardiac output. A part of the reduced vascular conductance may be attributed to a constriction of arteriovenous anastomoses.

Chapter 5

Inhibition of nitric oxide biosynthesis and carotid arteriovenous anastomotic shunting in the pig

Summary

The role of nitric oxide (NO) biosynthesis in the regulation of blood flow through arteriovenous anastomoses was evaluated in the carotid circulation of the anaesthetized pig. For this purpose, the effect of intracarotid (i.c.) administration of the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 0.1, 0.3 and 1.0 mg kg^{-1}; n=6) or saline (n=6) was studied on the distribution of common carotid blood flow, using the radioactive microsphere method.

Apart from the highest dose, L-NAME caused no major changes in the systemic haemodynamic variables. Both cardiac output and systemic vascular conductance were reduced by L-NAME (1 mg kg^{-1}), being reversed partly by L-arginine (100 mg kg^{-1}, i.c.). In both groups, L-arginine slightly reduced mean arterial blood pressure.

Total common carotid artery blood flow as well as its distribution over the capillary and arteriovenous anastomotic fraction remained stable after saline injections. In contrast, L-NAME caused a dose-dependent decline in common carotid artery blood flow and conductance and this decline was confined entirely to its arteriovenous anastomotic part.

Subsequent intracarotid injection of L-arginine (100 mg kg^{-1}) reversed the reduction in total carotid conductance almost completely and that in the arteriovenous anastomotic part partially. Additionally, L-arginine increased capillary conductance significantly in the L-NAME as well as the saline treated animals.

These results indicate that the L-arginine-NO pathway contributes little to the regulation of tissue perfusion in the porcine carotid circulation. In contrast, NO seems to play an important role in shunting arterial blood through arteriovenous anastomoses in the anaesthetized pig.

Based on: Van Gelderen E.M. and Saxena P.R. (1994). Inhibition of nitric oxide biosynthesis and carotid arteriovenous anastomotic shunting in the pig. Br. J. Pharmacol. 111; 961-967.

5.1 Introduction

Arteriovenous anastomoses have been found in animals and man, although their precise physiological function is not yet fully understood. Cutaneous arteriovenous anastomoses may be implicated in the regulation of the body temperature and blood pressure (Hales & Molyneux, 1988). Furthermore, dilatation of cranial arteriovenous anastomoses has been proposed to contribute to the pathogenesis of migraine headache (Heyck, 1969; Saxena, 1978, 1990).

Little is known of the mechanisms involved in opening arteriovenous anastomoses, which are normally under a constrictor tone. However, for several reasons nitric oxide (NO), which is formed from L-arginine by the calcium/calmodulin dependent enzyme NOS (Palmer *et al.*, 1988; Bredt & Snyder, 1990; Förstermann *et al.*, 1991), may be a potential candidate: (i) as indicated by the inhibition of NO biosynthesis using N^G-nitro-L-arginine methyl ester (L-NAME), continuous production of NO apparently contributes to the regulation of arterial blood pressure and tissue perfusion (Gardiner *et al*, 1990; Rees *et al.*, 1990; this thesis, chapter 2); (ii) compounds that act via the NO system (e.g. nitroglycerine, isosorbide mononitrate) cause headache as well as temporal artery dilatation in humans (Iversen, 1992; Iversen *et al.*, 1992); and (iii) as shown in chapter 4, systemic administration of L-NAME in anaesthetized pigs decreased the number of microspheres detected in the lungs after left atrial injection (i.e. bronchial plus systemic arteriovenous anastomotic blood flow); however, in these experiments cardiac output was also concomitantly reduced (this thesis, chapter 4).

The present investigation was devoted to study the effect of L-NAME, administered directly into the common carotid artery to avoid changes in cardiac output, on the distribution of the common carotid artery blood flow into arteriovenous and capillary (tissue) parts in the anaesthetized pig. Furthermore, jugular venous nitrite content was analyzed to establish whether the inhibition of NO biosynthesis is reflected by a reduction in plasma nitrite (NO_2) and nitrate (NO_3) levels *in vivo*; NO is rapidly decomposed to finally yield nitrite and nitrate, both detected in biological fluids (Green *et al.*, 1982; Furchgott *et al.*, 1990; Kelm & Schrader, 1990). Preliminary data from the present investigation were presented at the winter meeting of the British Pharmacological Society (Van Gelderen & Saxena, 1993).

5.2 Methods

General

Twelve domestic pigs (Yorkshire x Landrace; 15-18 kg) were anaesthetized with azaperone (120 mg, i.m.) and metomidate (150 mg, i.v.), intubated and connected to a respirator (Bear 2E, BeMeds AG, Baar, Switzerland) for intermittent positive pressure ventilation with a mixture of room air and oxygen. Arterial blood gas values were kept within physiological range (pH 7.39-7.49; pCO_2 37-44; pO_2 100-130) by adjusting oxygen supply, respiratory rate and tidal volume. Anaesthesia was maintained by continuous i.v. infusion of pentobarbitone sodium (20 mg kg^{-1} h^{-1} during the first hour followed by 12 mg kg^{-1} h^{-1}) in the right femoral vein. A catheter was introduced into the aortic arch via the left femoral artery, connected to a pressure transducer (Statham P23, Hato Rey, Puerto Rico) for measurement of arterial blood pressure and blood sample withdrawal to determine blood gases (ABL-510, Radiometer, Copenhagen, Denmark). Cardiac output and pulmonary arterial blood pressure were measured with a 6F-Swan-Ganz thermodilution catheter (Corodyn, Braun Melsungen AG, Melsungen, Germany) introduced into the pulmonary artery via the left femoral vein and connected to a cardiac output computer (WTI, Rotterdam, The Netherlands) as well as to a pressure transducer. Mean arterial (MAP) and mean pulmonary artery (MPAP) pressures were calculated from respective systolic (SBP) and diastolic (DBP) blood pressures as: MAP or MPAP= DBP+(SBP-DBP)/3. Heart rate was counted from the blood pressure signals. Systemic and pulmonary vascular conductances were calculated by dividing cardiac output by MAP and MPAP, respectively, with the assumption that the pulmonary capillary wedge pressure remained stable throughout the experiment. The right common carotid artery was dissected free and the total carotid blood flow at the ipsilateral side was measured with a flow probe (internal diameter: 2.5 or 3 mm) connected to a sine-wave electromagnetic flow meter (Transflow 600-system, Skalar, Delft, The Netherlands). Two hub-less needles (external diameter 0.5 mm), connected to a polyethylene tubing, were inserted into the common carotid artery against the direction of the blood flow for administration of radioactive microspheres or drugs. The right jugular vein was cannulated in order to collect venous blood samples to determine venous blood gases and to analyze nitrite levels. All catheters were filled with a heparin sodium solution (80 I.U. ml^{-1}) to prevent blood clotting. During the experiment, body temperature was kept above 37°C

and physiological saline was infused to compensate for fluid loss. The animals were allowed to stabilize for at least 45 min before the start of experiments.

Distribution of the common carotid blood flow

The distribution of the common carotid blood flow was measured using repeated injections of radioactive microspheres (mean±s.d. diameter: 15±1 μm; NEN Company, Dreieich, Germany) with different labels (^{141}Ce, ^{113}Sn, ^{103}Ru, ^{95}Nb or ^{46}Sc) (Johnston & Saxena, 1978). A suspension of approximately 250,000 microspheres was vortexed before each measurement and injected into the common carotid artery against the direction of the blood flow to ensure uniform mixing. At the end of the experiment the animals were killed and the heart, kidneys, lungs and the different cranial tissues were dissected out, weighed and put in vials. The radioactivity in these vials was counted for 10 min in a gamma-scintillation counter (Packard, Minaxi Autogamma 5000) using suitable windows for discriminating between the different isotopes.

Tissue blood flow (capillary blood flow) was calculated by multiplying the ratio between tissue radioactivity and the total radioactivity with the total carotid blood flow at the time of injection. Since radioactivity was absent in the heart and kidneys, all radioactive microspheres reaching the venous side of the circulation had passed through arteriovenous anastomoses to be ultimately sieved in the lungs. Therefore, the amount of radioactivity in the lungs served as an *index* for the arteriovenous anastomotic fraction of the common carotid blood flow.

Nitrite analysis

The nitrite content of venous blood samples, withdrawn from the ipsilateral jugular vein, was measured by a modification of the colorimetric procedure using the Griess reagent (0.5% sulfanilamide with 0.05% N-(naphthyl)-ethylenediamine in 2% H_3PO_4) (Green *et al.*, 1982). This assay is based on the diazotization of a sulfanilamide by nitrite and a subsequent coupling with N-(naphthyl)-ethylenediamine to yield a azochromophore which can be detected spectrophotometrically. Briefly, following centrifugation 150 μl of the supernatant was mixed with an equal amount of the Griess reagent in a 96-wells plate and incubated for 10 min at room temperature. Extinction was measured at 540 nm using a microplate reader (BIORAD, Model 3550) against a blank containing sulfanilamide. Sample nitrite concentration was calculated from a standard absorption curve constructed with several concentrations of sodium nitrite.

Since nitrite is converted rapidly to nitrate in the presence of oxygen samples were analyzed immediately after blood withdrawal.

Additionally, total nitrite *and* nitrate content in deproteinized samples was measured using Griess reagent following reduction of nitrates with a Klebsiella pneumoniae suspension (BBD 900990, RIVM-Pharmacology, Bilthoven, The Netherlands) into nitrites, detected by measuring the absorbance at 540 nm with a spectrophotometer (Perkin-Elmer lambda 15 UV/VIS) against a blank (Phizackerley & Al-Dabbagh, 1983).

Experimental protocol

After the stabilization period the animals were randomly assigned to either the treatment or control group, finally consisting of six animals each. At the start of the experiments, baseline haemodynamic parameters, venous nitrite levels, blood gases as well as the distribution of the common carotid blood flow was measured. Thereafter, the first group was treated with three consecutive doses of L-NAME (0.1, 0.3 and 1.0 mg kg^{-1}, i.c.), whereas an equal volume of saline was injected three times into the control animals group. In both groups, L-arginine (100 mg kg^{-1}, i.c.) was injected after the last dose of L-NAME or saline. Ten minutes after each dose all parameters were reassessed and blood samples collected.

Data presentation and analysis

All data, calculated with a personal computer (Olivetti PCS 286) using a set of specially developed computer programs (Saxena *et al.*, 1980), are presented in the text as mean±s.e. mean. The effect of treatment was analyzed in each group using a repeated measurement analysis of variance. When the samples represented different populations, the values after the different treatments were compared to baseline values by use of a Duncan's new multiple range test. Subsequently, the changes to baseline values among the two groups were tested using a Student's *t* test. Statistical significance was accepted at a level of $p \leq 0.05$.

Chemicals

The chemicals used in this study were: azaperone, metomidate (both from Janssen Pharmaceutica, Beerse, Belgium),L-arginine,N-(naphthyl)-ethylenediamine,N^G-nitro-L-arginine methyl ester (L-NAME), sulfanilamide (All from Sigma Chemical Co., St. Louis, U.S.A.), pentobarbitone sodium (Narcovet, Apharmo, Arnhem, The Netherlands)

and heparin sodium (Thromboliquine, Organon Teknika B.V., Boxtel, The Netherlands). Both L-arginine and L-NAME were dissolved in saline. All doses refer to their respective salts.

5.3 Results

Systemic haemodynamics

The systemic haemodynamic variables in both groups at baseline, followed by either saline or L-NAME (0.1, 0.3 and 1.0 mg kg^{-1}, i.c.), and L-arginine (100 mg kg^{-1}, i.c.) are depicted in Table 5.1. There were no significant differences between the two groups in the systemic haemodynamic values at baseline, and no major changes were observed in the saline-treated animals. L-NAME also did not significantly affect heart rate or mean arterial blood pressure, but after the highest dose pulmonary artery pressure increased (18±5%) and cardiac output decreased (-20±3%) significantly. Consequently, the calculated systemic (21±6%) and pulmonary (37±7%) vascular conductances were significantly decreased at the highest dose of L-NAME (1 mg kg^{-1}). L-arginine (100 mg kg^{-1}, i.c.) reversed the L-NAME-induced increase in mean pulmonary artery pressure as well as the reduction in systemic vascular conductance, and it also moderately lowered mean arterial blood pressure in both groups.

Carotid haemodynamics

As shown in Figure 5.1, no significant effects were observed in either total carotid blood flow or its distribution in saline-treated animals. In contrast, L-NAME caused a dose-dependent decline in total common carotid artery blood flow (maximum change: -37±3%). This effect was confined entirely to the arteriovenous anastomotic part with little change in the capillary part. The effects of L-NAME were partially reversed by L-arginine.

The effects of saline and L-NAME on carotid vascular bed conductance are shown in Figure 5.2. While no changes were observed with saline, intracarotid injections of the three consecutive doses of L-NAME (0.1 to 1.0 mg kg^{-1}) elicited dose-dependent decreases in total carotid conductance; the maximum change following the highest dose was -41±4%. Coincident with this reduction a decrease in arteriovenous anastomotic conductance was observed (-64±2%), whereas capillary conductance was unaffected. Subsequent intracarotid injection of L-arginine (100 mg kg^{-1}) reversed the reduction

in total carotid conductance almost completely (93±16%) and in the arteriovenous anastomotic conductance partially (50±6%). Additionally, L-arginine increased capillary conductance significantly in the L-NAME treated animals (+50±18%), whereas in the saline treated animals the conductance was increased when compared to baseline (+55±18%; Figure 5.2).

Table 5.1. Systemic haemodynamic variables measured at baseline and after intracarotid injections of saline (control; n=6) or L-NAME (n=6), followed by L-arginine (100 mg kg^{-1}).

		Saline or L-NAME (mg kg^{-1})			
	Baseline	0.1	0.3	1.0	L-Arginine
HR (beats min^{-1})					
Saline	93±7	91±6	91±6	91±6	91±6
L-NAME	104±5	102±5	100±6	94±3	92±4
MAP (mmHg)					
Saline	96±5	95±7	94±6	90±5	82±4†
L-NAME	100±2	100±5	104±6	109±8	91±6†
MPAP (mmHg)					
Saline	21.5±1.0	21.7±0.9	23.2±1.3	22.2±1.6	21.5±1.3
L-NAME	20.7±0.5	21.3±0.4	23.0±0.7	24.5±1.4*	21.2±0.7†
CO (l min^{-1})					
Saline	2.01±0.2	1.94±0.2	1.90±0.2	1.83±0.1	1.86±0.1
L-NAME	2.10±0.2	1.97±0.2	1.83±0.1	1.68±0.1*	1.80±0.1
SVC (ml min^{-1} $mmHg^{-1}$)					
Saline	21.3±2.7	20.9±2.2	20.4±1.7	20.7±1.7	22.7±1.1
L-NAME	21.1±1.9	19.6±1.4	17.7±1.6	16.2±1.0*	19.9±0.5†
PVC (ml min^{-1} $mmHg^{-1}$)					
Saline	96.3±14	89.6±7.0	81.8±3.5	84.0±7.2	87.0±4.1
L-NAME	102.5±10	92.6±9.8	80.7±7.0	70.6±8.9*	84.9±5.3

Data are given as mean±s.e. mean. *, $p \leq 0.05$ versus baseline; †, $p \leq 0.05$ L-arginine versus L-NAME (1.0 mg kg^{-1}) or saline. HR, heart rate; MAP, mean arterial blood pressure; MPAP, mean pulmonary artery pressure; CO, cardiac output; SVC, systemic vascular conductance; PVC, pulmonary vascular conductance.

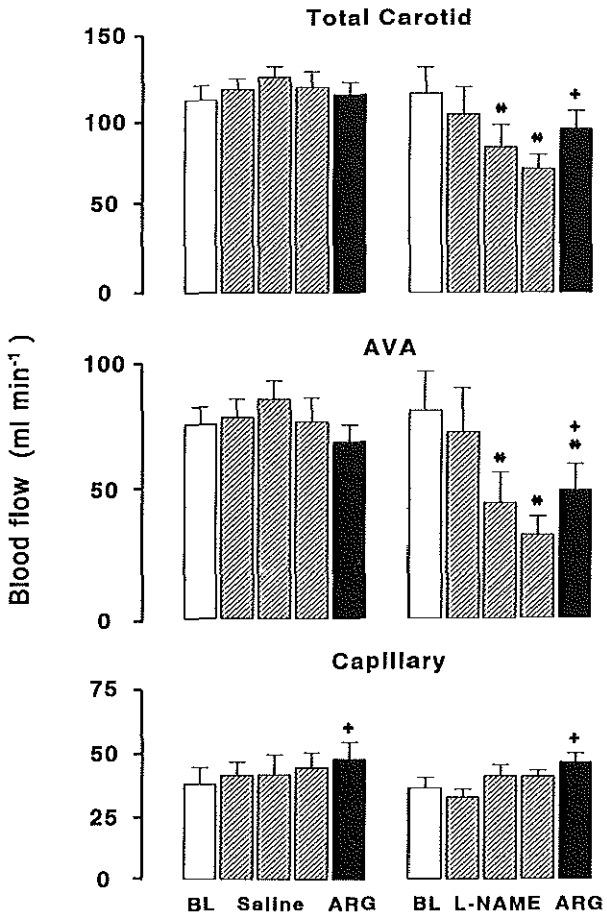

Figure 5.1 Total carotid, arteriovenous (AVA) and capillary blood flow at baseline (BL), after the three intracarotid injections with either saline (left panels; n=6) or L-NAME (right panels; 0.1, 0.3 and 1.0 mg kg^{-1}; n=6) and following L-arginine (Arg, 100 mg kg^{-1}; solid columns). In each set of panels, the hatched columns represent three increasing doses of saline or L-NAME. Values are given as mean±s.e. mean. +, $p \leq 0.05$ versus BL; *, $p \leq 0.05$ versus saline.

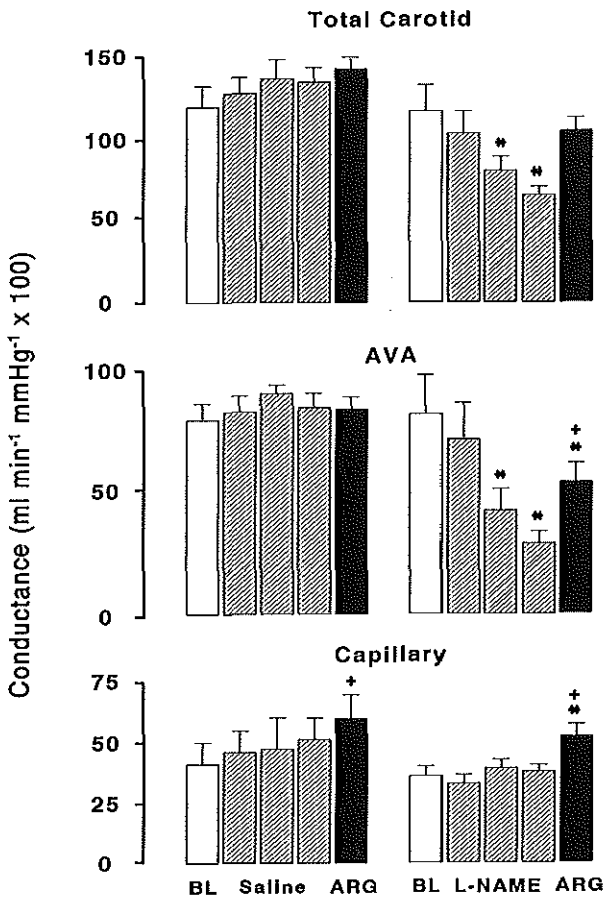

Figure 5.2 Total carotid, arteriovenous (AVA) and capillary vascular conductance at baseline (BL), after the three intracarotid injections with either saline (left panels; n=6) or L-NAME (right panels; 0.1, 0.3 and 1.0 mg kg^{-1}; n=6) and following L-arginine (Arg, 100 mg kg^{-1}; solid columns). In each set of panels, the hatched columns represent three increasing doses of saline or L-NAME. Values are given as mean±s.e. mean. +, $p \leq 0.05$ versus BL; *, $p \leq 0.05$ versus saline.

Regional carotid blood flow

Although L-NAME failed to alter total capillary blood flow, tissue blood flow to the eyes was reduced by L-NAME (0.3 and 1.0 mg kg^{-1}); the maximum changes observed from baseline (21±4 ml min^{-1} 100 g^{-1}) were 52±7% and 43±17%, respectively. No significant changes were observed in all other dissected extracerebral tissues. L-arginine restored blood flow to the eyes (16±2 ml min^{-1} 100 g^{-1}). As depicted in Figure 5.3, total brain flow increased following L-NAME (261±106%), which was unaffected by L-arginine. A further subdivision of the brain revealed a similar increase in all parts, also unaffected by L-arginine.

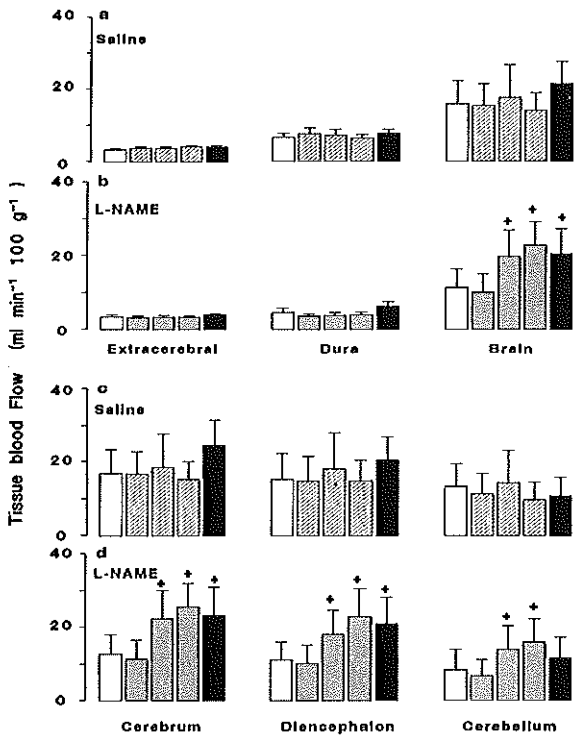

Figure 5.3 Blood flow distribution to cerebral and extracerebral tissues and to dura mater at baseline (open columns), after the three intracarotid injections with either saline (a, c; n=6) or L-NAME (b, d; 0.1, 0.3 and 1.0 mg kg^{-1}; n=6) and following L-arginine (Arg, 100 mg kg^{-1}; solid columns). In each set of panels, the hatched columns represent three increasing doses of saline or L-NAME. Values are given as mean±s.e. mean. +, $p \leq 0.05$ versus baseline.

Similarly, L-NAME failed to alter total capillary conductance, but reduced vascular conductance in the eyes significantly by 54±6% (baseline: 1.6±0.3 ml min^{-1} $mmHg^{-1}$ x 100) following L-NAME (0.3 and 1.0 mg kg^{-1}), whereas no significant changes were observed in all other dissected extracerebral tissues. L-arginine reversed the vascular conductance in the eyes (1.3±0.2 ml min^{-1} $mmHg^{-1}$ x 100). Furthermore, as shown in Figure 5.4, total brain conductance was enhanced by L-NAME (maximum: 212±77%), which was unaffected by L-arginine. A further subdivision of the brain revealed a similar increase in all parts, also being unaffected by L-arginine (Figure 5.4).

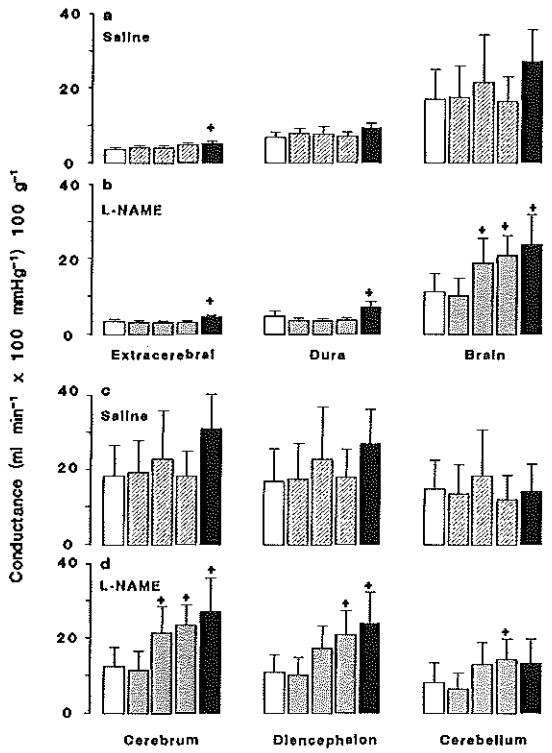

Figure 5.4 Vascular conductance in cerebral and extracerebral tissues and dura mater at baseline (open columns), after three intracarotid injections with either saline (a, c; n=6) or L-NAME (b, d; 0.1, 0.3 and 1.0 mg kg^{-1}; n=6) and following L-arginine (Arg, 100 mg kg^{-1}; solid columns). In each set of panels, the hatched bars represent three increasing doses of saline or L-NAME. Values are given as mean±s.e. mean. +, $p \leq 0.05$ versus baseline.

Arteriovenous oxygen saturation difference and nitrite content

In both groups arterial oxygen tension and carbon dioxide tension remained stable throughout the experiments. The difference in arteriovenous oxygen saturation increased significantly following the highest dose of L-NAME with 92±33%, whereas no changes were observed in the saline treated control group (Table 5.2). Similarly, a reduction in venous oxygen pressure was observed with this dose (19±4%), whereas venous carbon dioxide pressure remained stable. At baseline, total venous nitrite and nitrate content was 125±20 nmol ml^{-1} (n=12; 6 each in saline and L-NAME groups), which remained stable throughout the experiment in both saline and L-NAME treated animals (Table 5.2). At baseline, plasma nitrite content varied largely between saline and L-NAME treated animals; the pooled nitrite level was 3.3±0.6 nmol ml^{-1} (n=8; 4 each in saline and L-NAME groups). The nitrite level remained essentially unchanged in both groups, though with L-NAME (1.0 mg kg^{-1}; n=4) some decrease (34±19%) was observed compared to baseline values. L-arginine restored venous nitrite levels fully in the L-NAME treated animals, but was without effect in the control group (Table 5.2).

5.4 Discussion

Systemic haemodynamics and distribution of the common carotid blood flow

Using the radioactive microsphere technique, we have previously shown that inhibition of NO biosynthesis in the anaesthetized pig by systemic administration of L-NAME reduces blood flow and vascular conductance in a number of tissues as well as the microsphere content of the lungs (i.e. a sum of bronchial artery and total systemic arteriovenous anastomotic flows) (this thesis, chapter 4). In these experiments the reduction in arteriovenous anastomotic blood flow was not unequivocal and, moreover, it was accompanied by a decline in cardiac output. Therefore, in the present study we evaluated the effects of local intra-arterial injections of L-NAME (0.1, 0.3 and 1 mg kg^{-1}) on the porcine carotid vascular bed, where arteriovenous anastomoses are abundant (Saxena & Verdouw, 1982). Indeed, the effects of i.c. application of L-NAME were largely confined to the carotid vascular bed, since little changes in systemic haemodynamic variables were observed; only the highest dose significantly affected mean pulmonary artery pressure, cardiac output and systemic and pulmonary vascular conductances. It is unlikely that these changes were due to the carotid

Table 5.2. Jugular venous blood pO_2 (V pO_2), pCO_2 (V pCO_2), difference in arterial and jugular venous oxygen saturation (A-V O_2 sat) and jugular venous blood nitrite levels, both as nitrite alone (NO_2) and as nitrate and nitrite (NO_x) measured at baseline and after intracarotid injection of either saline or L-NAME and following L-arginine (100 mg kg^{-1}).

		Saline or L-NAME (mg kg^{-1})			
	Baseline	0.1	0.3	1.0	L-arginine
V pO_2 (mmHg)					
Saline	58±3	56±4	54±5	59±4	53±5
L-NAME	60±5	60±8	57±6	47±3*	50±4
V pCO_2 (mmHg)					
Saline	43±2	46±6	45±2	42±2	44±2
L-NAME	43±1	39±2	43±1	45±2	43±2
A-V O_2 sat (%)					
Saline	15.2±3	17.6±4	19.1±4	14.9±3	19.3±4
L-NAME	15.5±4	16.4±6	17.4±5	24.8±4*	18.5±4
NO_x (nmol ml^{-1})					
Saline	134±34	135±28	127±29	120±28	116±23
L-NAME	117±23	93±29	133±32	116±26	128±27
NO_2 (nmol ml^{-1})					
Saline	2.4±1	2.7±1	2.7±1	2.6±1	2.3±1
L-NAME	4.2±1	3.8±1	4.4±1	3.1±1†	3.5±1

Data are given as mean±s.e. mean. *, $p \leq 0.05$ versus corresponding saline value; †, $p \leq 0.05$ versus baseline value (n=4).

haemodynamic effects of L-NAME and, therefore, it seems that pharmacologically effective concentrations of L-NAME were present in the systemic circulation following its highest i.c. dose. These changes as well as the ability of L-arginine to reverse them are in accordance with our previous observations following systemic administration of L-NAME (this thesis, chapter 4).

Independent of any systemic haemodynamic effect, total common carotid blood flow and conductance were decreased by L-NAME. Moreover, it is remarkable that L-NAME caused no change in the capillary part of the carotid circulation and that the reductions in carotid blood flow and conduction were entirely confined to the

arteriovenous anastomotic part. The carotid arteriovenous anastomoses have been localized, as demonstrated by simultaneous injections of radioactive microspheres of different sizes, particularly in the skin, ears and dura mater, but also in the eyes and tongue (Saxena & Verdouw, 1985; Den Boer *et al.*, 1992). Although arteriovenous anastomoses are anatomically distinct from the rest of the microvasculature, light and electron microscopy studies have shown the presence of an endothelial layer in both human and rabbit peripheral arteriovenous anastomoses (Gorgas *et al.*, 1977; Fujiwara & Iijima, 1990). The finding that L-arginine, the endogenous substrate for NO-synthase, partly reversed the L-NAME-induced reductions in the carotid arteriovenous anastomotic blood flow and conductance suggests that NO, released from the endothelium, may provide a vasodilator tone in these specialized vessels. Interestingly, it has also been reported that sodium nitroprusside, which releases NO spontaneously (Feelisch & Noack, 1987), tends to increase systemic arteriovenous anastomotic shunt flow in anaesthetized rabbits (Hof & Hof, 1989).

Regional carotid blood flow

Total capillary and extracerebral blood flow were unaffected by L-NAME and L-arginine caused no significant increases in blood flows to or conductances in the various tissues, though total capillary conductance was increased by the latter in both saline and L-NAME treated animals. It therefore seems that, unlike the arteriovenous anastomoses, a NO-dependent vascular tone is absent in the capillary part of the cranial circulation of the anaesthetized pig. However, it may be noted that under pentobarbitone anaesthesia capillary blood flow is approximately one-third of the total carotid blood flow (Saxena & Verdouw, 1982, 1985; Den Boer *et al.*, 1992; present results) and thus the basal release of NO may already be too low to detect a significant decrease.

In contrast to a number of studies which reported a moderate reduction in cerebral blood flow following NO-synthase inhibition, suggesting a basal NO-release in this part of the cranial circulation (Kovách *et al.*, 1992 ; Prado *et al.*, 1992; Seligsohn & Bill, 1993), in our present experiments L-NAME failed to reduce the cerebral component of carotid blood flow. The absence of an endothelial NO-dependent dilator tone is supported by the recent finding that cultured rat brain microvessel endothelial cells do not show basal release of NO (Durieu-Trautmann *et al.*, 1993). Moreover in our experiments, L-NAME increased blood flow to the brain as well as to its constituent parts. This increase cannot be explained by hypercapnia, since arterial

carbon dioxide tension remained unchanged. One possible explanation may be a redistribution of blood from the constricted arteriovenous anastomoses towards the cerebral blood vessels. If this is the case, it is of interest that the redistribution is solely confined to the brain tissue without affecting extracerebral blood flow. Since the increase in cerebral blood flow was not susceptible to L-arginine, a NO-independent mechanism seems likely. Finally, the inability of L-NAME to reduce cerebral blood flow is probably not due to the low doses used, since 1 mg kg^{-1} of L-NAME has been shown to block NO-production, as reflected by the increase in mean arterial blood pressure and blood flow reductions in a number of peripheral tissues (e.g. Gardiner *et al.*, 1990; this thesis, chapter 2 and 4). In doses up to 30 mg kg^{-1}, both L-NAME and L-NNA are without effect on brain blood flow (this thesis, chapter 2; Faraci & Heistad, 1992), though in higher doses these agents can significantly reduce cerebral blood flow (Kovách *et al.*, 1992; Prado *et al.*, 1992; Seligsohn & Bill, 1993). It is tempting to speculate that at high doses NOS inhibitors may act at a different site within the brain. Apart from the endothelium, NOS is abundantly present in neural tissues including perivascular nerves surrounding cerebral vessels (Bredt *et al.*, 1990; Nozaki *et al.*, 1993). The activity of these nerves, denoted as nitroxidergic, is inhibited by L-NNA (Toda *et al.*, 1993) and topical application of L-NMMA reduces the diameter of cerebral arteries (Faraci, 1991). Hence, it is conceivable that these NOS inhibitors do not readily pass the blood brain barrier and can only inhibit the activity of nitroxidergic nerves when used in high doses.

Changes in arteriovenous oxygen saturation difference and nitrite content

L-NAME caused an increase in arteriovenous oxygen saturation difference which is in keeping with an effect on arteriovenous anastomoses. A reduced difference between arterial and venous oxygen has been observed by Heyck (1969) in migraine patients and the difference is increased by ergot alkaloids together with a reduction in arteriovenous anastomoses blood flow (Johnston & Saxena, 1978; Den Boer *et al.*, 1992). Since no changes were observed following L-NAME in the capillary fraction of the carotid blood flow, it is unlikely that the increase in percentage difference in arteriovenous oxygen saturation is due to an enhanced oxygen consumption in the capillaries.

Concomitant with the decrement in arteriovenous anastomotic blood flow by L-NAME, a reduction of the plasma nitrite content in jugular venous blood was observed, which was restored by endogenous substrate L-arginine. Though this finding

is in line with the proposed competition of L-NAME and L-arginine for the enzyme NOS, basal plasma nitrite content was within the low micromolar range and a marked difference between the saline and L-NAME group was observed. Since NO may bind to hemeproteins or be converted into nitrite and nitrate it is not clear if this is a true reflection of the basal endothelial NO release. In contrast, total nitrite *and* nitrate content was approximately 40 times higher than nitrite levels and no changes were observed following L-NAME. In human plasma, an approximately 3-fold lower nitrate level was measured by Wennmalm and co-workers (1992), together with low nitrite concentrations. These authors suggested that NO is converted predominantly within red blood cells to nitrate. If this is the case, it is still unclear to which extent endothelial NO release contributes to the plasma nitrate content. Therefore, NOS inhibition may not be *per se* reflected by a reduction in nitrate levels. However, the inability of L-NAME to reduce nitrate levels in jugular venous blood samples may be explained in part by the reduced renal blood flow observed following L-NAME (this thesis, chapter 4). An augmented nitrate elimination due to a reduced renal perfusion may oppose the expected reduction in plasma nitrate levels.

In conclusion, the results of the present investigation indicate that, under resting conditions, the L-arginine-NO pathway has little contribution to the regulation of tissue perfusion within the carotid circulation of the anaesthetized pig. In contrast, NO may participate in controlling shunting of arterial blood through arteriovenous anastomoses.

Chapter 6

The effect of nitric oxide donors on haemodynamics and blood flow distribution in the porcine carotid circulation

Summary

The role of nitric oxide (NO) in the regulation of capillary and arteriovenous anastomotic blood flow was evaluated in the carotid circulation of the pig. For this purpose, the effect of intracarotid (i.c.) infusions of saline and two NO donors, nitroprusside sodium (NPR) and S-nitroso-N-acetylpenicillamine (SNAP) in concentrations of 3-100 $\mu g\ min^{-1}$ was studied on systemic haemodynamics and carotid blood flow and its distribution in anaesthetized pigs with low arteriovenous anastomotic blood flow, by use of the radioactive microsphere method.

Apart from heart rate, which increased after both NPR and SNAP, no major changes in systemic haemodynamic variables were observed. In contrast to saline, both NPR and SNAP increased common carotid blood flow, vascular conductance and vascular pulsations dose-dependently.

The distribution of the carotid artery blood flow over capillary and arteriovenous anastomotic fraction remained stable after saline infusions. Both NPR and SNAP enhanced total capillary blood flow and conductance. In contrast to NPR, arteriovenous anastomotic blood flow and conductance were increased by SNAP. At the tissue level, capillary blood flow increases following NPR or SNAP were reflected by an increase in both extracerebral and dural blood flow without changes in total brain blood flow.

These results indicate that both NO donors cause arteriolar dilatation together with enhanced vascular pulsations in the carotid circulation of the pig. Probably by way of a 'steal' phenomenon, this pronounced arteriolar dilatation limits the effect of NO donors on arteriovenous anastomoses. The finding of the present investigation support the contention that dilatation of intra- and extracranial arteries and arterio-

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venous anastomoses leads to increased vascular pulsations, which (rather than increased blood flow *per se*) could, at least in part, be responsible for the headache caused by nitrovasodilators.

6.1 Introduction

Nitric oxide (NO) released from endothelial cells by numerous stimuli participates in the regulation of vascular tone (see Moncada *et al.*, 1991; this thesis, chapter 1). A dilatation of large (extra) cranial arteries and arteriovenous anastomoses has been implicated in the pathogenesis of migraine, though the mechanism involved is not fully clear (Saxena, 1978; Humphrey & Feniuk, 1991; Ferrari & Saxena, 1993; Lance, 1993). Recent observations in both animals and man have led to the suggestion that NO may also be involved in migraine. Inhibition of NO-biosynthesis resulted in a reduced blood flow through porcine systemic and cranial arteriovenous anastomoses, thus indicating a NO-dependent vasodilator tone in these shunt vessels (this thesis, chapter 4 and 5). Histamine, known to relax isolated cranial blood vessels via release of NO from vascular endothelium (Toda, 1990; Fujiwara *et al.*, 1992), induced pulsatile headache in migraine patients (Krabbe & Olesen, 1980). Nitrovasodilators, such as nitroglycerin and isosorbide mononitrate, which are generally thought to act as NO-donor, caused dose-dependent temporal artery dilatations and headache in man (Iversen, 1992; Iversen *et al.*, 1992). Moreover, patients with migraine appeared to be more sensitive to nitroglycerin as demonstrated by a more pronounced headache and cerebral artery dilatation relative to non-migraine subjects (Olesen *et al.*, 1993; Thomsen *et al.*, 1993).

The present investigation was devoted to study the effect of NO donors on the carotid haemodynamics and the distribution of the common carotid artery blood flow over arteriovenous and capillary (tissue) fractions in the anaesthetized pig. Some reports suggest that S-nitrosothiols may account, as an intermediate, for the NO-induced vasorelaxant actions (Ignarro, 1990; Myers *et al.*, 1990; Rubanyi *et al.*, 1991). Therefore, we investigated both sodium nitroprusside (NPR), known to release NO spontaneously (Feelisch & Noack, 1987a), and the NO-'precursor' S-nitroso-N-acetylpenicillamine (SNAP; Ignarro *et al.*, 1981). To preserve the constrictor tone in cranial arteriovenous anastomoses, necessary to study mechanisms involved in opening

of arteriovenous anastomoses, a recently described anaesthetic regimen was used (Den Boer *et al.*, 1993). Some of these results were presented at the winter meeting of the British Pharmacological Society (Van Gelderen *et al.*, 1994).

6.2 Methods

General

Domestic pigs (Yorkshire x Landrace; 15-17 kg) were anaesthetized with i.m. injection of ketamine (25 mg kg^{-1}) and midazolam (0.3 mg kg^{-1}). Following injection of thiopentone (6 mg kg^{-1}), the animals were intubated and connected to a respirator (Bear 2E, BeMeds AG, Baar, Switzerland) for intermittent positive pressure ventilation with a mixture of room air and oxygen. By adjusting oxygen supply, respiratory rate and tidal volume, arterial blood gas values were kept within physiological range (pH 7.39-7.49; pCO_2 37-44; pO_2 100-130). Anaesthesia was maintained by a continuous i.v. infusion with a mixture of fentanyl (18-38 μg kg^{-1} h^{-1}) and thiopental (6-12 mg kg^{-1} h^{-1}). Due to differences in sensitivity of individual animals, infusion rate was adjusted in each animal using two criteria: (i) absence of blinking reflex, indicative of appropriate anaesthesia and (ii) venous oxygen saturation values below 60%, indicative of low arteriovenous anastomotic blood flow.

A catheter was introduced into the aortic arch via the left femoral artery, connected to a pressure transducer (Statham P23, Hato Rey, Puerto Rico) for measurement of arterial blood pressure and blood sample withdrawal to determine blood gases (ABL-510, Radiometer, Copenhagen, Denmark). Cardiac output and pulmonary arterial blood pressure were measured with a 6F-Swan-Ganz thermodilution catheter (Corodyn, Braun Melsungen AG, Melsungen, Germany) introduced into the pulmonary artery via the left femoral vein and connected to a cardiac output computer (WTI, Rotterdam, The Netherlands) as well as to a pressure transducer. Mean arterial (MAP) and mean pulmonary artery (MPAP) pressures were calculated from respective systolic (SBP) and diastolic (DBP) blood pressures as: MAP or MPAP = DBP+ (SBP-DBP)/3. Heart rate was triggered from the blood pressure signals using a tachograph as well as counted directly from these signals over a 30 s recording interval. Systemic vascular conductance was calculated by dividing cardiac output by MAP. The right common carotid artery was dissected free and the total carotid blood

flow on the ipsilateral side was measured with a flow probe (internal diameter: 2.5 or 3 mm) connected to a sine-wave electromagnetic flow meter (Transflow 600-system, Skalar, Delft, The Netherlands). Vascular pulsations were calculated from the flow signals as the difference in systolic and diastolic blood flow values. Two hubless needles (external diameter 0.5 mm), bent at right angles and connected to a polyethylene tubing, were inserted into the common carotid artery against the direction of the blood flow for administration of radioactive microspheres or drugs. The right jugular vein was cannulated to collect venous blood samples to determine venous blood gases. All catheters were filled with a heparin sodium solution (80 I.U. ml^{-1}) to prevent blood clotting. During the experiment, physiological saline was infused to compensate for fluid loss and body temperature was kept between 37-38°C. The animals were allowed to stabilize for at least 45 min before the start of experiments.

Distribution of common carotid artery blood flow

The distribution of common carotid artery blood flow was measured using repeated injections of radioactive microspheres (mean±s.d. diameter: 15±1 μm; NEN Company, Dreieich, Germany) with different labels (^{141}Ce, ^{113}Sn, ^{103}Ru, ^{95}Nb or ^{46}Sc) (Johnston & Saxena, 1978). A suspension of approximately 250,000 microspheres was vortexed before each measurement and injected into the common carotid artery against the direction of the blood flow to ensure uniform mixing. At the end of the experiment the animals were killed and the heart, kidneys, lungs and the different intra- and extracranial tissues were dissected out, weighed and put in vials. The radioactivity in these vials was counted for 10 min in a γ-scintillation counter (Packard, Minaxi Autogamma 5000) using suitable windows for discriminating between the different isotopes. Radioactivity was counted in entire tissues, except in case of bone, fat and skin, where aliquots (more than 50% of total weight) were analyzed.

Tissue (capillary) blood flow was calculated by multiplying the ratio of tissue and total radioactivity by total carotid blood flow at the time of injection. Since radioactivity was absent in the heart and kidneys, all radioactive microspheres reaching the venous side of the circulation had passed through arteriovenous anastomoses to be ultimately sieved in the lungs (see Johnston & Saxena, 1978; Saxena & Verdouw, 1982). Therefore, the amount of radioactivity in the lungs represents the arteriovenous anastomotic fraction of common carotid blood flow.

Experimental protocol

After the stabilization period the animals were randomly assigned to either control or treatment groups. At the start of the experiments, baseline haemodynamic parameters, blood gases as well as the distribution of the common carotid artery blood flow was measured. Thereafter, the first group was treated four times with i.c. infusions of saline (control; n=6). The second group was treated with either three (10, 30, 100 $\mu g\ min^{-1}$; n=4) or four increasing doses of NPR (3, 10, 30 and 100 $\mu g\ min^{-1}$; n=4). In the third group, the potential effect of the solvent (20% v/v ethanol in saline) was measured, followed by three doses of SNAP (3, 10, 30 $\mu g\ min^{-1}$; n=6). Additionally, in this group three animals were treated with SNAP (100 $\mu g\ min^{-1}$) to determine the effect on carotid and systemic haemodynamic values. The amount of ethanol infused during the experimental period (75 min) ranged from 0.97 to 1.1 ml, depending on the weight of the animal. Since solvent infusion did not induce significant effects, SNAP induced responses were compared with responses observed in the saline (control) group. In all groups infusion rate (125 $\mu l\ min^{-1}\ 15\ kg^{-1}$) was adjusted to correct for differences in animal weight. Each dose was infused for 15 min in which time period the carotid blood flow signal and systemic haemodynamic parameters stabilized; during the last five minutes of each infusion all parameters were reassessed and blood samples collected.

Data presentation and analysis

All data, calculated with a personal computer (Olivetti PCS 286) using a set of specially developed computer programs (Saxena *et al.*, 1980), are presented in the text as mean ± s.e. mean. The effect of treatment was analyzed in each group using a repeated measurement analysis of variance. When the samples represented different populations, the values after the different treatments were compared to baseline values by use of Duncan's new multiple range test. Subsequently, the percent changes from baseline values among the groups were tested using the Duncan's test for multiple comparisons. Statistical significance was accepted at a level of $p \leq 0.05$.

Chemicals

The chemicals used in this study were: fentanyl citrate (Janssen Pharmaceutica, Beerse, Belgium), heparin sodium (Heparin Leo, LEO Pharmaceutical Products B.V., Weesp, The Netherlands), ketamine HCl (Apharmo, Arnhem, The Netherlands), midazolam (Dormicum, Roche, Mijdrecht, The Netherlands), nitroprusside sodium

(Pharmacy Department, University Hospital Dijkzigt, Rotterdam, The Netherlands), S-nitroso-N-acetylpenicillamine (SNAP; RBI, Natick, USA), thiopental sodium (Rhône-Poulenc Rorer, Amstelveen, The Netherlands). All chemicals were dissolved in sterile saline, except fentanyl citrate and SNAP. Fentanyl citrate was dissolved in propylene glycol and subsequently diluted in distilled water. SNAP was dissolved in a mixture containing 20% v/v ethanol (70%) in saline to reach a concentration of 100 μg 125 μl^{-1} (highest dose used). This stock solution was subsequently diluted with sterile saline to obtain the lower concentrations (3, 10 and 30 μg ml^{-1}). All doses refer to their respective salts.

Table 6.1 Systemic haemodynamic variables measured at baseline and after intracarotid infusions with saline (control; n=6), nitroprusside sodium (NPR; n=8, except 3 μg min^{-1} n=4) and solvent (S) followed by S-nitroso-N-acetylpenicillamine (SNAP;n=6, except 100 μg min^{-1} n=3).

		Saline, NPR or SNAP (μg min^{-1})				
	Baseline	S	3	10	30	100
HR (beats min^{-1})						
Saline	77±7	-	77±7	79±8	81±9	81±10
NPR	85±5	-	87±10	88±5	98±4	130±12*†
SNAP	80±8	81±8	87±10	100±19	117±19†	124±27†
MAP (mmHg)						
Saline	94±5	-	93±6	93±6	91±6	89±7
NPR	91±5	-	90±5	86±5	81±5	74±6†
SNAP	82±3	84±6	88±7	85±6	74±6	74±10
MPAP (mmHg)						
Saline	21±1.4	-	22±1.2	22±1.2	22±1.3	22±1.4
NPR	23±0.6	-	22±1.5	22±1.0	22±0.9	20±1.0
SNAP	20±0.8	21±1	21±0.7	20±1.1	19±1.3	20±1.9
CO (l min^{-1})						
Saline	1.4±0.1	-	1.4±0.1	1.4±0.2	1.4±0.2	1.5±0.2
NPR	1.7±0.2	-	1.7±0.3	1.7±0.2	1.8±0.2	1.9±0.2
SNAP	1.4±0.1	1.3±0.1	1.3±0.1	1.3±0.1	1.4±0.1	1.9±0.2
SVC (ml min^{-1} $mmHg^{-1}$)						
Saline	15±1	-	15±1	15±2	15±2	16±1
NPR	19±2	-	19±4	21±3	22±2	26±3
SNAP	17±2	16±2	15±1	16±3	19±1	26±2

Data are given as mean±s.e. mean. †, $p \leq 0.05$ versus baseline; *, $p \leq 0.05$ versus saline. HR, heart rate; MAP, mean arterial blood pressure; MPAP, mean pulmonary artery pressure; CO, cardiac output; SVC, systemic vascular conductance.

6.3 Results

Systemic haemodynamics

The systemic haemodynamic variables of all groups at baseline and followed by either saline, solvent, NPR or SNAP are depicted in Table 6.1. There were no significant differences between the three groups in systemic haemodynamic values at baseline. Neither saline nor solvent (ethanol/saline, 20% v/v) induced any change in the systemic haemodynamic variables. Both NPR (100 μg min^{-1}) and SNAP (30 and 100 μg min^{-1}) increased heart rate significantly; with the highest dose heart rate increased by 57±15% and 44±17%, respectively. Mean arterial blood pressure tended to decrease following NPR and SNAP, being significantly different from baseline values with the highest dose of NPR only. Neither drug significantly affected other systemic haemodynamic variables.

Carotid haemodynamics

Carotid blood flow remained stable throughout the saline infusions (Figure 6.1). Likewise, no changes were observed in baseline carotid blood flow (52±6 ml min^{-1}) in the SNAP group following solvent infusion (51±8 ml min^{-1}; not shown in figure). Both NPR and SNAP induced a dose-dependent increase in carotid blood flow. The maximum increase from baseline, observed with the highest dose (100 μg min^{-1}) of the two compounds, was 79±9% and 85±18%, respectively. The increase in carotid blood flow was associated partly with enhanced vascular pulsations, which were increased from baseline by a maximum of 59±17% (NPR) and 109±45% (SNAP) (Figure 6.1). At baseline, 23±4% (n=20) of the total carotid blood flow was shunted through arteriovenous anastomoses and no differences between groups were observed. The extent of shunting in pigs anaesthetized with fentanyl and thiopental is much less than that (~75%) previously observed in pigs anaesthetized with pentobarbital (Saxena & Verdouw, 1982; Den Boer *et al.*, 1990; Villalón *et al.*, 1990; this thesis chapter 5). The NPR-induced carotid blood flow increase was reflected by a dose-dependent increase in total capillary blood flow; maximum increase from baseline was 100±11%. No changes were observed in the arteriovenous anastomotic fraction (Figure 6.2). In contrast to NPR, SNAP increased both total capillary blood flow and arteriovenous anastomotic blood flow. Total capillary blood flow was increased by 71±19% from baseline, being significantly different from saline treated animals from 10 μg min^{-1} onwards.

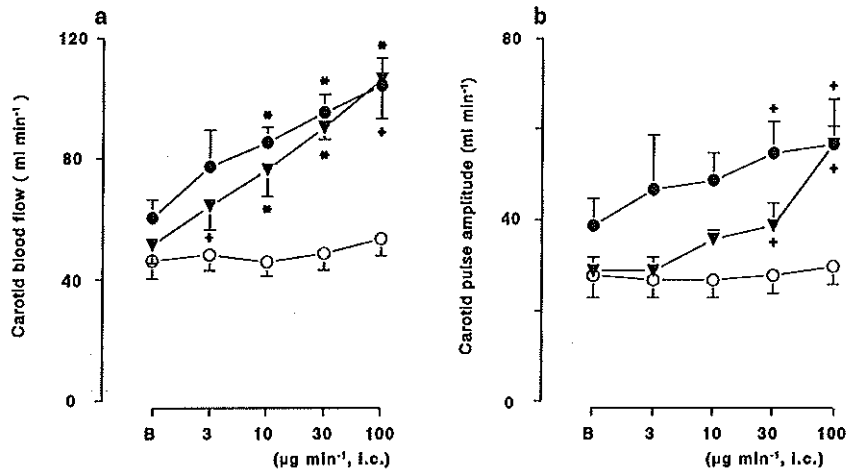

Figure 6.1 Carotid blood flow (a) and pulse amplitude (b) at baseline (B) and after intracarotid infusions with either saline (125 µl min^{-1}, 4 times; n=6), nitroprusside sodium (NPR; 3 µg min^{-1}, n=4, and 10, 30 and 100 µg min^{-1}, n= 8 each) or S-nitroso-N-acetyl-penicillamine (SNAP; 3, 10, 30 µg min^{-1}; n=6 each, and 100 µg min^{-1}; n=3). In case of SNAP solvent values (not shown) were not different from baseline values. In both panels, (○), (●) and (▼) represent saline, NPR and SNAP, respectively. Values are given as mean±s.e. mean. †, P≤0.05 versus baseline (B); *, P≤0.05 versus saline.

Arteriovenous anastomotic blood flow increased significantly from baseline following SNAP (10 and 30 µg min^{-1}); maximum increase (245±151%) was observed with the highest dose (Figure 6.2).

Both NPR and SNAP increased carotid vascular conductance from baseline by a maximum of 127±15% and 178±66%, respectively; being significantly different from the saline group from 10 µg min^{-1} onwards (Figure 6.3). Arteriovenous anastomotic conductance was essentially unaffected by NPR (maximum increase 72±40%; n.s.), whereas SNAP enhanced the conductance significantly from baseline by 284±160%. Total capillary conductance was equally increased by NPR and SNAP; 30 µg min^{-1} of either NPR or SNAP increased conductance by 89±7% and 91±21%, respectively (Figure 6.3).

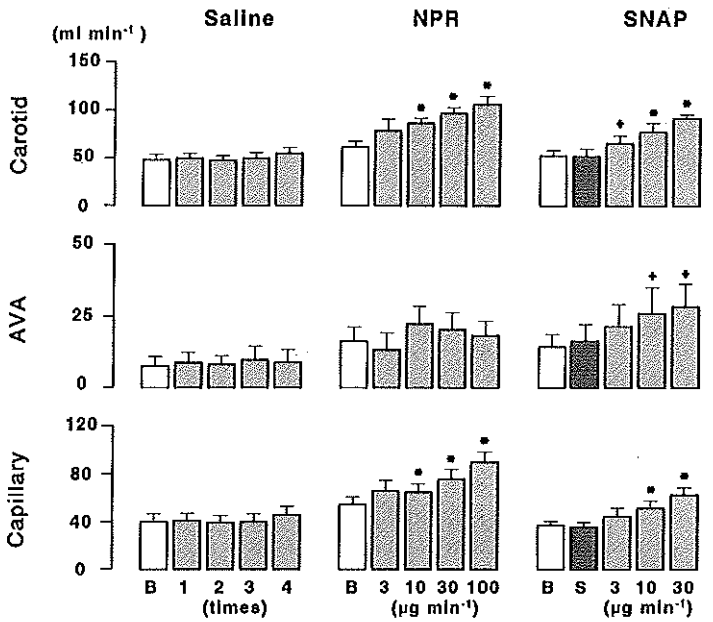

Figure 6.2 Total carotid, arteriovenous anastomotic (AVA) and capillary blood flow at baseline (B) and after intracarotid infusions with either saline (125 µl min^{-1}, 4 times; n=6), nitroprusside sodium (NPR; 3 µg min^{-1}; n=4, and 10, 30 and 100 µg min^{-1}; n=8 each) or S-nitroso-N-acetylpenicillamine (SNAP; 3, 10 and 30 µg min^{-1}; n=6). In each set of panels, open and hatched bars represent baseline values and values after increasing doses of saline, NPR or SNAP, respectively; in case of SNAP cross-hatched bars represent values after the solvent (S). Data are given as mean±s.e. mean. †, p≤0.05 versus baseline; *, p≤0.05 versus saline.

Regional carotid blood flow

As depicted in Figure 6.4, neither saline infusions nor solvent infusion affected blood flow distribution to either the total brain, extracerebral or dural tissues. Both NPR and SNAP failed to affect total brain blood flow. In contrast, a significant increase in extracerebral blood flow was observed following NPR (10, 30 and 100 µg min^{-1}) and SNAP (10 and 30 µg min^{-1}); maximum increases were 106±12% and 84±17%, respectively. Dural blood flow was enhanced dose-dependently by NPR, 30 and 100 µg min^{-1}, with a maximum increase of 368±96%. Likewise, SNAP (10 and 30 µg min^{-1}) enhanced dural blood flow from baseline (154±57%).

A number of extracerebral tissues (bones, ear, muscle and skin) showed enhanced blood flows following both NPR and SNAP (Figure 6.5). Furthermore, SNAP increased blood flow to the fat (Figure 6.5) as well as the eyes from a baseline value of 30±5 ml min^{-1} to a maximum of 75±20 ml min^{-1} with the highest dose (100 μg min^{-1}).

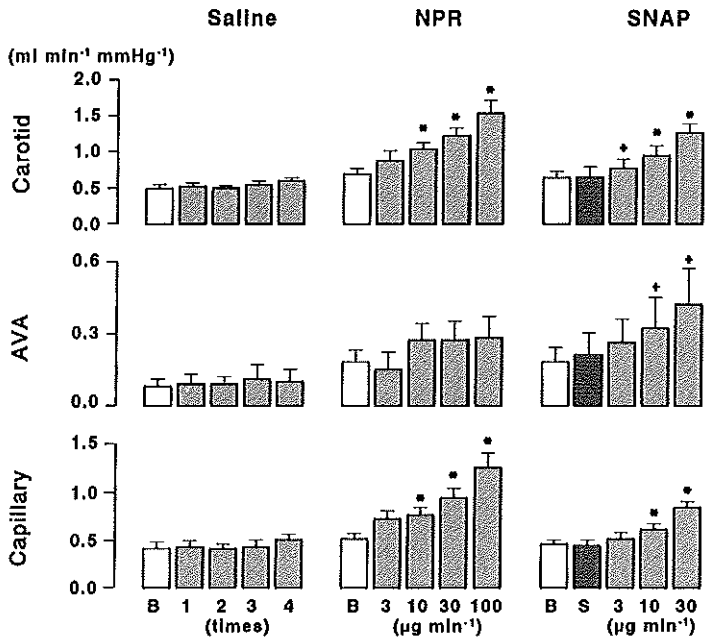

Figure 6.3 Total carotid, arteriovenous anastomotic (AVA) and capillary conductance at baseline (B) and after intracarotid infusions with either saline (125 μl min^{-1}, 4 times; n=6), nitroprusside sodium (NPR; 3 μg min^{-1}; n=4, and 10, 30 and 100 μg min^{-1}; n=8 each) or S-nitroso-N-acetylpenicillamine (SNAP; 3, 10 and 30 μg min^{-1}; n=6). In each set of panels, open and hatched bars represent baseline values and values after increasing doses of saline, NPR or SNAP, respectively; in case of SNAP cross-hatched bars represent values after the solvent (S). Data are given as mean±s.e. mean. †, $p \leq 0.05$ versus baseline; *, $p \leq 0.05$ versus saline.

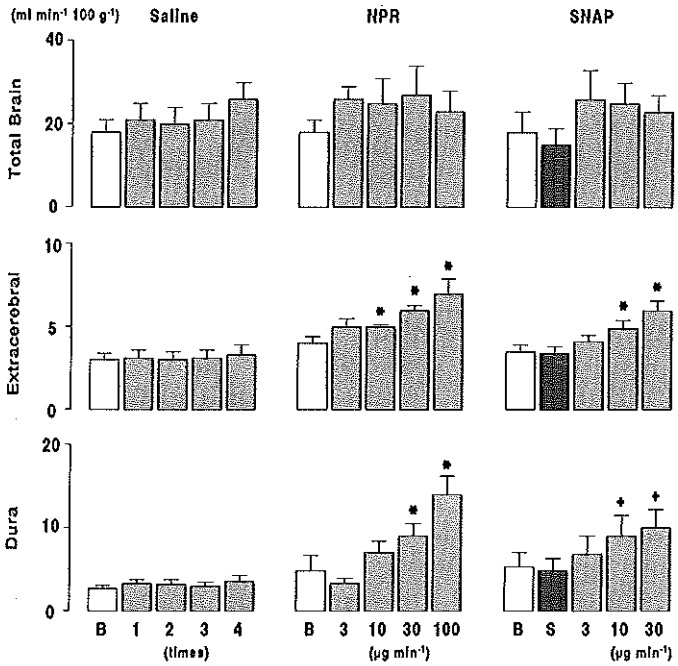

Figure 6.4 Blood flow distribution to cerebral and extracerebral tissues and to dura mater at baseline (B) and after intracarotid infusions with either saline (125 µl min^{-1}, 4 times; n=6), nitroprusside sodium (NPR; 3 µg min^{-1}; n=4, and 10, 30 and 100 µg min^{-1}; n=8 each) or S-nitroso-N-acetylpenicillamine (SNAP; 3, 10 and 30 µg min^{-1}; n=6). In each set of panels, open and hatched bars represent baseline values and values after increasing doses of saline, NPR or SNAP, respectively; in case of SNAP cross-hatched bars represent values after the solvent (S). Data are given as mean±s.e. mean. †, $p \leq 0.05$ versus baseline; *, $p \leq 0.05$ versus saline.

Arteriovenous oxygen saturation difference

In all groups venous oxygen and carbon dioxide tension remained stable throughout the experiments. In contrast to saline, NPR and SNAP reduced the arterial partial oxygen pressure by 24±2% and 9±2%, respectively (Table 6.2). The difference in arteriovenous oxygen saturation was reduced from baseline with the highest dose of NPR, whereas no changes were observed in either saline- or SNAP-treated animals (Table 6.2).

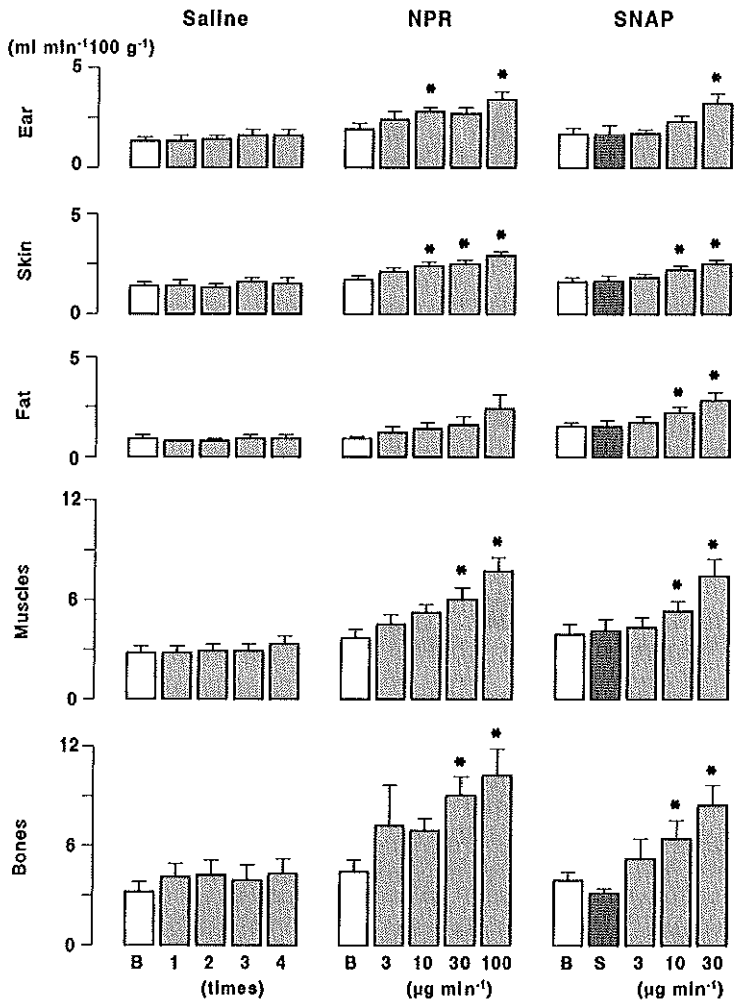

Figure 6.5 Blood flow to extracerebral tissues at baseline (B) and after intracarotid infusions with either saline (125 µl min^{-1}, 4 times; n=6), nitroprusside sodium (NPR; 3 µg min^{-1}; n=4, and 10, 30 and 100 µg min^{-1}; n=8 each) or S-nitroso-N-acetylpenicillamine (SNAP; 3, 10 and 30 µg min^{-1}; n=6). In each set of panels, open and hatched bars represent baseline values and values after increasing doses of saline, NPR or SNAP, respectively; in case of SNAP cross-hatched bars represent values after the solvent (S). Data are given as mean±s.e. mean. *, $p \leq 0.05$ versus baseline.

Table 6.2 Arterial pO_2 (*A* pO_2), jugular venous blood pO_2 (*V* pO_2) and difference in arterial and jugular venous oxygen saturation, measured at baseline and after intracarotid injection of either saline (control; n=6), nitroprusside sodium (NPR; n=8, except 3 μg min^{-1} n=4) and solvent (S) followed by S-nitroso-N-acetylpenicillamine (SNAP; n=6).

			Saline, NPR or SNAP (μg min^{-1})			
	Baseline	S	3	10	30	100
A pO_2 *(mmHg)*						
Saline	121±8	-	123±7	121±7	117±7	114±8
NPR	117±4	-	119±3	109±4	103±4†	89±3†
SNAP	115±4	116±3	118±5	109±2	104±3†	n.d.
V pO_2 *(mmHg)*						
Saline	37±3	-	38±2	39±2	38±2	37±2
NPR	35±2	-	39±5	37±2	38±2	41±3
SNAP	41±6	39±5	42±6	40±6	42±5	n.d.
A-V O_2 *sat (%)*						
Saline	45±7	-	42±7	41±6	42±7	43±6
NPR	50±5	-	45±10	42±4	40±4	35±4†
SNAP	38±9	38±8	36±9	37±8	34±7	n.d.

Data are given as mean±s.e. mean. n.d.: not determined. †, $p \leq 0.05$ versus baseline value.

6.4 Discussion

General

This study aimed to investigate the effect of exogenous NO on the carotid circulation of the anaesthetized pig with low arteriovenous anastomotic blood flow. In the conscious state arteriovenous anastomoses are under a predominantly sympathetic constrictor tone and, as a consequence, less than 5% of intracardially injected microspheres are sieved in the lungs (Van Woerkens *et al.*, 1990). During anaesthesia, arteriovenous shunting is increased substantially limiting comparison with conscious animals. Recently, a mixture of fentanyl and thiopental was reported to preserve tone in arteriovenous anastomoses enabling the investigation of effects on arteriovenous anastomoses under conditions which resemble the conscious state (Den Boer *et al.*, 1993). In accord with the latter study, we observed that approximately 20% of the carotid blood flow was directed to arteriovenous anastomoses. It is to be noted that, as seems to be the case in humans (Heyck, 1969), basal arteriovenous shunting is

higher in the carotid vascular bed than in the systemic vascular bed, which may be explained by the relatively greater skin area, together with higher density of arteriovenous anastomoses in the head skin, perfused by the carotid circulation (Saxena & Verdouw, 1985).

Systemic and carotid haemodynamics

Following intracarotid infusions of NPR and SNAP, systemic haemodynamic variables were not much affected indicating that the drug effects were primarily confined to the carotid part of the circulation. However, we can not fully exclude a systemic contribution as heart rate increased (NPR: 100 $\mu g\ min^{-1}$; SNAP: 30 and 100 $\mu g\ min^{-1}$) and MAP tended to decrease with NPR. The increase in heart rate is most likely reflex mediated to compensate for the expected fall in systemic blood pressure, although additional effects may be involved. For example, hypercapnia is associated with tachycardia presumably via activation of chemoreceptors in the carotid body. Interestingly, NO synthase-containing fibres have been demonstrated in the carotid body of rats (Wang *et al.*, 1993; Höhler *et al.*, 1994) and cats (Prabhakar *et al.*, 1993). Moreover, NPR increases cGMP formation in the carotid body arterioles (Wang *et al.*, 1991), whereas inhibition of the endogenous NO biosynthesis increases chemoreceptor outflow and reduces cGMP formation (Prabhakar *et al.*, 1993). So far, it is not yet known whether exogenous NO stimulates chemoreceptors to reduce their outflow.

Both NO donors induced similar increases in carotid blood flow which were accompanied by enhanced vascular pulsations. Likewise, enhanced pulsatility was observed in the human carotid and cerebral artery using the NO donor nitroglycerin (MacDonald *et al.*, 1989; Thomsen *et al.*, 1993). The mechanism underlying these enhanced pulsations is not clear, but it may be related to changes in vascular compliance following vasodilatation. Since pulsatility is also increased by calcitonin gene-related peptide (MacDonald *et al.*, 1989; this thesis chapter 7), an exclusively NO-dependent phenomenon is precluded.

Carotid blood flow distribution

Both NPR and SNAP showed a similar arteriolar dilatation as reflected by the increase in capillary blood flow and conductance, whereas in contrast to NPR, only SNAP enhanced blood flow through arteriovenous anastomoses. The latter finding is in accord with our previous observations demonstrating that endogenous NO may

participate in the regulation of shunt flow in the carotid circulation (this thesis chapter 5). However, the lack of effect on arteriovenous anastomoses with NPR is in divergence with this observation and the previously reported tendency of NPR to increase blood flow through systemic arteriovenous anastomoses in rabbits (Hof & Hof, 1989). This discrepancy may be explained partly by the concomitant rise in capillary blood flow which could lead to a redirection of carotid blood flow towards the capillaries to negate the increase in arteriovenous anastomotic blood flow ('steal' phenomenon). Recent experiments in humans (Bruning, 1994) have shown that NPR, infused into the brachial artery but in a much lower concentration (5 ng kg^{-1} min^{-1}), also failed to increase arteriovenous anastomotic blood flow in the fingers.

The differences observed in the present experiments between NPR and SNAP may be explained by differences in the amount of NO released from these compounds. At 37°C, approximately 0.2 µmol min^{-1} is released spontaneously from NPR (Feelisch & Noack, 1987a), whereas under similar conditions SNAP releases approximately six times more NO (Kodja *et al.*, 1994). Moreover, it has been suggested that nitrosothiols may be actively degraded at vascular membranes (Kowaluk & Fung, 1990) and SNAP has been shown to have an *in vitro* chemical half life of 70 min (Mathews & Kerr, 1993), although the rate of formation and degradation may differ significantly in biological fluids. Therefore, it is conceivable that following repeated infusions SNAP accumulates at arteriovenous anastomoses, thus providing sufficient local concentration of NO to cause vasodilatation.

A reduced difference between arterial and venous oxygen saturation has been observed in migraine patients (Heyck, 1969), suggesting enhanced shunting of arterial blood towards the venous side of the circulation. Likewise, after inhibition of NO synthesis this difference was increased together with a reduction in blood flow through arteriovenous anastomoses in anaesthetized pigs (this thesis chapter 5). In the present study, the ability of SNAP to enhance arteriovenous blood flow is not reflected by a concomitant reduction in the difference in arteriovenous oxygen saturation. Since both NPR and SNAP induced a marked arteriolar dilatation, an increase in oxygen consumption at the capillary level may partially account for this lack of effect. Additionally, a reduced arterial oxygen supply may have limited the amount of oxygen shunted towards the venous side. Indeed, a significant reduction in partial oxygen pressure together with a slight reduction in arterial oxygen saturation was observed with NPR and SNAP.

Regional carotid blood flow distribution

Both NO donors enhanced extracerebral blood flow without changing brain blood flow, suggesting that these drugs do not interfere with autoregulatory processes. However, the absence of cerebral blood flow changes does not *per se* rule out middle cerebral artery dilatation as reported in migraine (Friberg *et al.*, 1991), because such large vessel dilatations can not be detected with radioactive microspheres. Although, the role of extracranial dilatation in migraine headache is often disregarded, dilatation and increased pulsations in the extracranial blood vessels have been observed on the side of headache (Ray & Wolff, 1940; Iversen *et al.*, 1990). Manual pressure as well as ergotamine oppose these effects and relieve pain (Graham & Wolff, 1938; Drummond & Lance, 1988).

NPR and SNAP enhanced dural blood flow significantly. Since this tissue may be the source of headache pain (Ray & Wolff, 1940), and dural blood vessels are innervated by perivascular trigeminal sensory nerves (Mayberg *et al.*, 1984), it is tempting to speculate that enhanced blood flow together with pronounced vascular pulsations in this tissue participate in vascular headache. However, it must be pointed out that several antimigraine drugs (ergotamine, dihydroergotamine and sumatriptan) failed to affect porcine dural blood flow and dural arteriovenous anastomotic shunting (Den Boer *et al.*, 1992a), and, in contrast to perivascular administration, i.v. administration of sumatriptan did not constrict feline pial artery, but increased carotid vascular resistance (Connor *et al.*, 1992).

In conclusion, the present findings demonstrate that the NO donors, NPR and SNAP, induce arteriolar dilatation together with enhanced vascular pulsations in the carotid circulation of the pig. Though arteriovenous anastomotic blood flow did increase with SNAP, the pronounced arteriolar dilatation appears to limit the effect of exogenous NO on arteriovenous anastomoses, probably due to a 'steal' phenomenon. Taken together, these findings support the opinion that dilatation of (extra)cranial arteries and arteriovenous anastomoses may lead to increased vascular pulsations, which (rather than increased blood flow) could be partly responsible for the headaches caused by nitrovasodilators.

Chapter 7

Carotid blood flow distribution, haemodynamics and inotropic responses following calcitonin gene-related peptide in the pig

Summary

The sensory neuropeptide, calcitonin gene-related peptide (α-CGRP), has been implicated in the pathogenesis of migraine headache. This study aimed to evaluate the effects of intracarotid infusions of human α-CGRP (10, 30 and 100 pmol kg^{-1} min^{-1}; n=8), as compared to those of saline (four times; n=8) on haemodynamics and blood flow distribution within the carotid circulation of the anaesthetized pig, using the radioactive microsphere method. Furthermore, the effects of antimigraine drugs, dihydroergotamine (100 μg kg^{-1}, i.v; n=4) or sumatriptan (300 μg kg^{-1}, i.v.; n=4), on these parameters were studied in the presence of the infusion of the highest concentration of human α-CGRP. Additionally, putative positive inotropic responses to human α-CGRP (10^{-9}-10^{-7} M) were investigated in porcine isolated atrial and ventricular trabeculae. Human α-CGRP increased carotid artery blood flow and conductance dose-dependently, together with an enhancement in vascular pulsations. These effects were associated with a fall in systemic blood pressure with concomitant increases in heart rate and cardiac output. The increase in carotid blood flow was reflected by an increase in total capillary blood flow, predominantly to extracerebral tissues including the dura, whereas blood flow through arteriovenous anastomoses remained stable. Both dihydroergotamine and sumatriptan reduced carotid blood flow and its capillary fraction without affecting systemic vascular conductance. In tissues, these drugs reversed blood flow increases due to human α-CGRP in most extracerebral tissues, but failed to reduce dural blood flow.

In porcine isolated atrial and ventricular trabeculae, noradrenaline (10^{-8}-10^{-5} M) increased force of contraction in a concentration dependent manner. In contrast, human α-CGRP (10^{-9}-10^{-7} M) failed to increase force of contraction in atrial trabeculae (n=6)

Based on: Van Gelderen E.M., Du X.Y., Schoemaker R.G. and Saxena P.R. (1995). Carotid blood flow distribution, haemodynamics and inotropic responses following calcitonin gene-related peptide in the pig. Eur. J. Pharmacol. 284; 51-60.

and exerted only a moderate concentration-dependent positive inotropic effect in ventricular trabeculae (~25% of the response to 10^{-5} M noradrenaline, n=10).

These data indicate that human α-CGRP caused arteriolar dilatation together with a fall in blood pressure in the pig. The tachycardia may be reflex-mediated, but the peptide also exerts a moderate positive inotropic action on ventricular trabeculae. The fall in systemic arterial blood pressure and the marked increase in capillary blood flow most likely prevented the opening of arteriovenous anastomoses. Furthermore, the antimigraine drugs, dihydroergotamine and sumatriptan, were able to reverse blood flow changes induced by human α-CGRP in the porcine carotid circulation.

7.1 Introduction

In spite of considerable debate concerning the precise mechanism, the pathogenesis of migraine is associated with dilatation of large (extra)cranial and arteriovenous anastomoses (Saxena, 1978; Drummond & Lance 1988; Ferrari & Saxena, 1993). Recent observations in animals and humans have suggested that both calcitonin gene-related peptide (CGRP) and nitric oxide (NO) may act in concert in the vascular events observed during migraine attacks. The 37-amino acid peptide, CGRP, is abundantly present in sensory fibres, including fibres innervating the heart and trigeminal vascular system (Mulderry *et al.*, 1985; Uddman *et al.*, 1986; Suzuki *et al.*, 1989). The peptide has been shown to be a potent vasodilator exerting differential regional haemodynamic effects (Brain *et al.*, 1985; Franco-Cereceda *et al.*, 1987; Gardiner *et al.*, 1989). In migraineurs, elevated plasma levels of CGRP have been observed in the extracerebral circulation during headache (Goadsby *et al.*, 1990) and antimigraine agents like dihydroergotamine and sumatriptan reduced elevated CGRP plasma levels in these patients, as well as in cats during trigeminal nerve stimulation (Goadsby & Edvinsson, 1993). Likewise, nitrovasodilators, which are generally thought to act by releasing NO (Ignarro, 1990), induce more pronounced headache (Olesen *et al.*, 1993) and cranial artery dilatation in migraine patients than in normal subjects (Thomsen *et al.*, 1993). Finally, nitrovasodilators increase blood flow through arteriovenous anastomoses in the carotid circulation of the pig (This thesis, chapter 6) and may activate perivascular sensory nerves to release CGRP in cerebral as well as cutaneous microvessels (Wei *et al.*, 1992; Holzer & Jočic, 1994).

The present study aimed to evaluate the effects of human α-CGRP on haemodynamics and blood flow distribution within the carotid circulation of the pig, using the radioactive microsphere method. Since dihydroergotamine and sumatriptan have been shown to reduce carotid blood flow in pigs (Den Boer *et al.*, 1992a,b), we also studied the effects of these two drugs on haemodynamics and carotid blood flow distribution during the infusion of human α-CGRP.

Additionally, α-CGRP has been shown to increase cardiac output (Lappe *et al.*, 1987; Wang *et al.*, 1989; Gardiner *et al.*, 1991) and positive inotropic responses have been described in isolated atria of various species (Saito *et al.*, 1986; Sigrist *et al.*, 1986; Franco-Cereceda *et al.*, 1987; Du *et al.*, 1994). As preliminary experiments demonstrated an enhanced cardiac output following human α-CGRP in pigs and as little is known about its putative inotropic effects in the pig heart, the effect of α-CGRP was investigated further in porcine isolated atrial and ventricular trabeculae.

A preliminary account of some of the results was presented at the XIIth International Congress of Pharmacology (Van Gelderen & Saxena, 1994).

7.2 Methods

The protocol of this study was submitted to and approved by the Institutional Committee for the use of animals.

General (in-vivo)

After an overnight fast, sixteen domestic pigs (Yorkshire x Landrace; 15-17 kg) were anaesthetized with i.m injection of ketamine (25 mg kg^{-1}) and midazolam (0.3 mg kg^{-1}). Following injection of thiopentone (6 mg kg^{-1}) in the middle ear vein, the animals were intubated and connected to a respirator (Bear 2E, BeMeds AG, Baar, Switzerland) for intermittent positive pressure ventilation. By adjusting oxygen supply, respiratory rate and tidal volume, arterial blood gas values were kept within the physiological range (pH 7.39-7.49; pCO_2 37-44; pO_2 100-130). Anaesthesia was maintained by a continuous i.v. infusion with a mixture of fentanyl (18-38 μg kg^{-1} h^{-1}) and thiopental (6-12 mg kg^{-1} h^{-1}). This anaesthetic regimen was used to preserve the vasoconstrictor tone in cranial arteriovenous anastomoses that is necessary to study mechanisms involved in opening of arteriovenous anastomoses (Den Boer *et al.*, 1993).

Due to differences in the sensitivity of individual animals, the infusion rate was adjusted in each animal using two criteria: (i) absence of blinking reflex, indicative of appropriate anaesthesia and (ii) venous oxygen saturation values below 60%, indicative of low arteriovenous anastomotic blood flow.

A catheter was introduced into the aortic arch via the left femoral artery and was connected to a pressure transducer (Statham P23, Hato Rey, Puerto Rico) for the measurement of arterial blood pressure as well as withdrawal of blood samples to determine blood gases (ABL-510, Radiometer, Copenhagen, Denmark). Cardiac output, pulmonary arterial blood pressure and body temperature were measured with a 6F-Swan-Ganz thermodilution catheter (Corodyn, Braun Melsungen AG, Melsungen, Germany) introduced into the pulmonary artery via the left femoral vein and connected to a cardiac output computer (WTI, Rotterdam, The Netherlands) as well as to a pressure transducer. Mean arterial (MAP) and mean pulmonary artery (MPAP) pressures were calculated from the respective systolic (SBP) and diastolic (DBP) blood pressures as: MAP or MPAP = DBP+(SBP-DBP)/3. Heart rate was recorded using a tachograph triggered from blood pressure signals as well as counted directly from these signals over a 30-s recording interval. Systemic vascular conductance was calculated by dividing cardiac output by mean arterial pressure. The right common carotid artery was dissected free and the total carotid blood flow was measured with a flow probe (internal diameter: 2.5 or 3 mm) connected to a sine-wave electromagnetic flow meter (Transflow 600-system, Skalar, Delft, The Netherlands). Carotid vascular conductance was calculated by dividing carotid blood flow by mean arterial pressure and vascular pulse amplitude was calculated from the flow signals as the difference in systolic and diastolic blood flow values. Two hubless needles (external diameter 0.5 mm), bent at right angles and connected to polyethylene tubing (internal diameter 0.5 mm), were inserted into the common carotid artery against the direction of the blood flow for administration of radioactive microspheres or drugs. The right jugular vein was cannulated to collect venous blood samples to determine venous blood gases. All catheters were filled with a heparin sodium solution (80 I.U. ml^{-1}) to prevent blood clotting. During the experiment, physiological saline was infused to compensate for fluid loss and body temperature was kept between 37 and 38°C.

Distribution of common carotid artery blood flow

As described extensively by Johnston and Saxena (1978), the distribution of common carotid artery blood flow was measured using repeated injections of radioactive microspheres (mean±s.d. diameter: 15±1 μm; NEN Company, Dreieich, Germany) with different labels (^{141}Ce, ^{113}Sn, ^{103}Ru, ^{95}Nb or ^{46}Sc). Briefly, a suspension of approximately 250,000 microspheres was vortexed and injected into the common carotid artery at baseline and following the various treatments. Microspheres were injected against the direction of the blood flow to ensure uniform mixing. At the end of the experiment, the animals were killed and the heart, kidneys, lungs and the different intra- and extracranial tissues were dissected out, weighed and put in vials. The radioactivity in these vials was counted for 10 min in a γ-scintillation counter (Packard, Minaxi Autogamma 5000) using suitable windows for discriminating between the different isotopes. Radioactivity was counted in whole tissues, except in the case of bone, fat and skin, where aliquots (more than 50% of total weight) were analyzed.

Tissue (capillary) blood flow was calculated by multiplying the ratio of tissue and total radioactivity by total carotid blood flow at the time of injection, using a set of computer programs (Saxena *et al.*, 1980). Since radioactivity was absent in the heart and kidneys, all radioactive microspheres reaching the venous side of the circulation had passed through arteriovenous anastomoses to be ultimately sieved in the lungs (see Johnston & Saxena, 1978; Saxena & Verdouw, 1982). Therefore, the amount of radioactivity in the lungs represents the arteriovenous anastomotic fraction of common carotid blood flow.

Isolated atrial and ventricular trabeculae

Cardiac tissues were obtained from pigs at the end of control (saline) experiments and atrial and ventricular trabeculae were set up for recording of contractions as described previously (Schoemaker *et al.*, 1992; Du *et al.*, 1994). Briefly, after excision, pieces of atrial and ventricular myocardium were placed in ice-chilled oxygenated Krebs buffer (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, $KHPO_4$ 1.2 and glucose 8.3) and atrial and ventricular trabeculae (<1 mm thickness) were carefully dissected free. The trabeculae were mounted in organ baths containing Krebs buffer (gassed with 95% O_2 and 5% CO_2; 37°C) and isometric tension was recorded with a Harvard transducer. Resting tension was set to 750 and

1950 mg for atrial and ventricular tissues, respectively, in order to provide optimal loading conditions. Tissues were paced at 1 Hz, using electrical field stimulation (3 ms, voltage 20% above threshold).

Experimental protocols

In vivo experiments

After a 45-min stabilization period, the animals were assigned at random to treatment (intracarotid infusions) with either saline (4 times, 125 $\mu l\ min^{-1}$, n=8) or human α-CGRP (10, 30 and 100 pmol $kg^{-1}\ min^{-1}$, n=8). Each dose was infused for 10 min to obtain stable blood flow signals. At baseline and after each infusion period, haemodynamic variables were collected and radioactive microspheres were injected. After the highest dose of human α-CGRP (100 pmol $kg^{-1}\ min^{-1}$), infusion was continued and either dihydroergotamine (100 $\mu g\ kg^{-1}$, i.v.; n=4) or sumatriptan (300 $\mu g\ kg^{-1}$, i.v.; n=4) was injected and all variables were reassessed after 10 min.

In vitro experiments

Baseline force of contraction was measured after stabilization, and inotropic responsiveness was assessed by making a concentration-response curve for noradrenaline (10^{-8}-10^{-5} M). The maximum concentration of noradrenaline was restricted to 10^{-5} M, since tissues were irreversibly damaged after higher concentrations (see Schoemaker *et al.*, 1992; Du *et al.*, 1994). Tissues with a response to 10^{-5} M noradrenaline smaller than 25 mg were excluded from further analysis.

After the tissues had been washed six times and allowed to stabilize, a concentration-response curve for human α-CGRP (10^{-9}-10^{-7} M) was obtained in both atrial and ventricular tissues. Responses to human α-CGRP were expressed as percentages of the response to 10^{-5} M noradrenaline. Following another wash and stabilization period of at least 10 min, a second noradrenaline concentration-response curve was made to check the viability of the tissues.

Data presentation and analysis

The effect of saline and human α-CGRP treatment in the two groups of anaesthetized pigs was analyzed by a repeated-measurement analysis of variance. When the samples represented different populations, the values after each treatment were compared to baseline values by using Duncan's new multiple range test. Subsequently, the percent changes from baseline values among groups were tested using

Student's *t*-test. Baseline values for isolated atrial and ventricular trabeculae were compared using an unpaired *t*-test. The effects of noradrenaline and human α-CGRP were analyzed with an analysis of variance for repeated measurements. In all cases, statistical significance was accepted at a level of $P \leq 0.05$.

Drugs

The compounds used were: dihydroergotamine mesylate (Wander-Pharma, Uden, The Netherlands), fentanyl citrate (Janssen Pharmaceutica, Beerse, Belgium), heparin sodium (Heparin Leo, LEO Pharmaceutical Products B.V., Weesp, The Netherlands), human α-CGRP (Saxon Biochemicals GMBH, Hannover, Germany), ketamine HCl (Apharmo, Arnhem, The Netherlands), midazolam (Dormicum, Roche, Mijdrecht, The Netherlands), noradrenaline bitartrate (Sigma, St. Louis, MO, U.S.A.), sumatriptan (Glaxo Research Group, Ware, U.K.), thiopental sodium (Rhône-Poulenc Rorer, Amstelveen, The Netherlands). All chemicals were dissolved in sterile saline, except fentanyl citrate which was dissolved in propylene glycol and subsequently diluted in distilled water.

7.3 Results

Haemodynamics

Systemic and carotid haemodynamic variables measured at baseline and after intracarotid infusions with either saline or human α-CGRP and following injections with either sumatriptan or dihydroergotamine in the presence of human α-CGRP infusion (100 pmol kg^{-1} min^{-1}) are depicted in Table 7.1. No differences were observed between the two groups at baseline, and infusion of saline caused no significant change in these variables. In contrast, human α-CGRP reduced mean arterial blood pressure with a maximum decrease of 31±4% (100 pmol kg^{-1} min^{-1}). Heart rate, cardiac output and systemic vascular conductance increased dose dependently with a maximum of 78±13%, 44±14% and 104±8%, respectively, whereas no change was observed in stroke volume. Carotid blood flow and carotid vascular conductance were enhanced following human α-CGRP with a maximum increase of 199±46% (30 pmol kg^{-1} min^{-1}) and 277±58% (100 pmol kg^{-1} min^{-1}), respectively (Figure 7.1; Table 7.1).

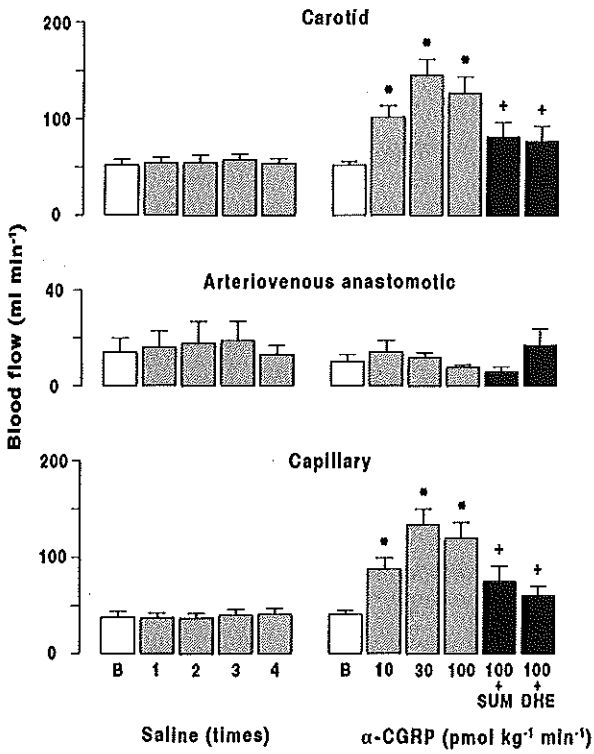

Figure 7.1 Carotid blood flow and its distribution over arteriovenous anastomotic and capillary fractions at baseline (B) and after intracarotid infusions of saline (four times, n=8; left panels) or human α-CGRP (10, 30 and 100 pmol kg^{-1} min^{-1}, n=8; right panels), followed by either sumatriptan (SUM; 300 μg kg^{-1}, i.v.; n=4) or dihydroergotamine (DHE; 100 μg kg^{-1}, i.v.; n=4) in the presence of human α-CGRP (100 pmol kg^{-1} min^{-1}). Data are given as mean ± s.e.mean. *, P≤0.05 versus saline values; +, P≤0.05 versus human α-CGRP.

Together with the enhanced carotid blood flow, a significant increase in vascular pulse amplitude was observed with human α-CGRP, 30 and 100 pmol kg^{-1} min^{-1}; maximum increase from baseline 142±19% (Table 7.1).

Intravenous injections of either sumatriptan (300 μg kg^{-1}) or dihydroergotamine (100 μg kg^{-1}) in the presence of intracarotid infusions of human α-CGRP (100 pmol kg^{-1} min^{-1}) reduced mean arterial blood pressure by 42±5% and 33±5%, respectively,

Table 7.1 Systemic and carotid haemodynamic variables measured at baseline and after intracarotid infusions of either saline (control; n=8) or human α-CGRP (n=8) and following injections of either sumatriptan (SUM, 300 μg kg^{-1} iv; n=4) or dihydroergotamine (DHE, 100 μg kg^{-1} iv; n=4) in the presence of human α-CGRP (100 pmol kg^{-1} min^{-1}).

		Saline or human α-CGRP (pmol kg^{-1} min^{-1})				
	Baseline	10	30	100	100+SUM	100+DHE
MAP (mmHg)						
Saline	86±4	83±4	85±4	85±4	84±4	-
α-CGRP	89±2	86±2	76±3*	61±3*	49±5†	52±4
HR (beats min^{-1})						
Saline	76±6	74±5	74±6	75±5	74±6	-
α-CGRP	80±10	90±11	104±9*	139±13*	114±24	134±18
CO (l min^{-1})						
Saline	13±01	13±01	13±01	13±01	14±02	-
α-CGRP	14±01	17±02	19±02*	19±02*	15±02	16±02
SV (ml)						
Saline	18±08	18±08	18±07	18±08	19±10	-
α-CGRP	18±15	19±17	18±17	15±24	15±30	13±30
SVC (ml min^{-1} $mmHg^{-1}$)						
Saline	16±1	16±1	16±1	16±1	16±2	-
α-CGRP	15±1	19±2	25±2*	31±2*	31±1	31±1
CVC (ml min^{-1} $mmHg^{-1}$.100)						
Saline	60±8	66±9	67±12	70±10	64±4	-
α-CGRP	58±4	118±13*	193±18*	207±20*	165±21†	145±23†
CFP (ml min^{-1})						
Saline	47±10	46±11	45±9	45±8	47±9	-
α-CGRP	67±7	74±6	108±9*	159±16*	148±7	181±34

Values represent means±s.e. mean; *, P≤0.05 versus saline; †, P≤0.05 versus human α-CGRP (100 pmol kg^{-1} min^{-1}). HR, heart rate; MAP, mean arterial blood pressure; CO, cardiac output; SV, stroke volume; SVC, systemic vascular conductance; CVC, carotid vascular conductance; CFP, carotid flow pulse.

but induced no further change in the other systemic haemodynamic variables (Table 7.1). Both carotid blood flow and carotid vascular conductance were reduced by sumatriptan (42±3% and 23±6%, respectively) and dihydroergotamine (33±5% and 26±4%, respectively) (Figure 7.1; Table 7.1). Carotid flow pulse remained stable following either drug (Table 7.1).

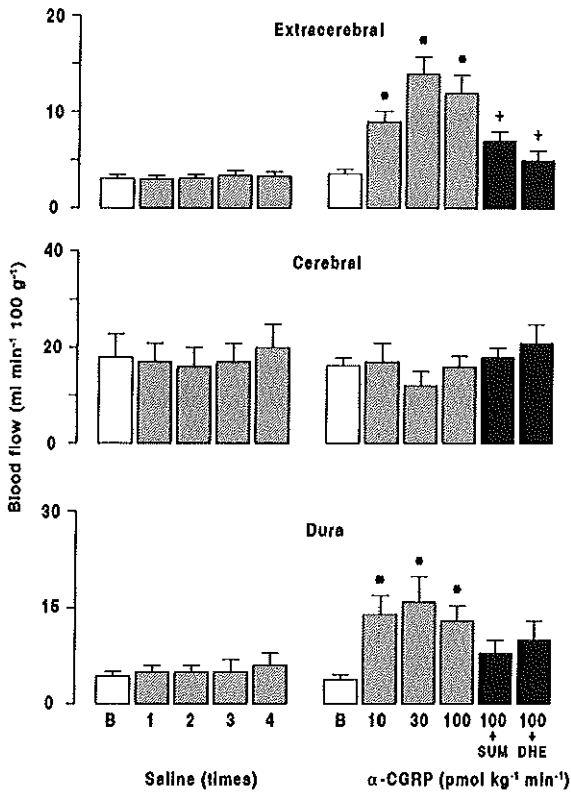

Figure 7.2 Tissue blood flow distribution over extracerebral and cerebral tissues at baseline (B) and after intracarotid infusions of saline (four times, n=8; left panels) or human α-CGRP (10, 30 and 100 pmol kg^{-1} min^{-1}, n=8; right panels), followed by either sumatriptan (SUM; 300 μg kg^{-1}, i.v.; n=4) or dihydroergotamine (DHE; 100 μg kg^{-1}, i.v.; n=4) in the presence of human α-CGRP (100 pmol kg^{-1} min^{-1}).

Carotid blood flow distribution

The human α-CGRP-induced increase in carotid blood flow was confined to the capillary fraction, which was increased from baseline (41±4 ml min^{-1}) by 237±45% (30 pmol kg^{-1} min^{-1}). At baseline, 21±5% (n=16) of the total carotid blood flow was directed towards arteriovenous anastomoses and no differences were observed between the two groups. Neither saline nor human α-CGRP induced changes in the arterio-

venous anastomotic fraction (Figure 7.1). Likewise, the increase in carotid vascular conductance was reflected by an increase in capillary conductance, which increased from baseline (46±4 ml min^{-1} $mmHg^{-1}$) to a maximum of 330±57% (data not shown). Sumatriptan and dihydroergotamine reduced equally the human α-CGRP-induced increase in capillary blood flow by 42±4% and 44±5%, respectively, whereas neither drug affected arteriovenous anastomotic blood flow (Figure 7.1). Similarly, sumatriptan and dihydroergotamine reduced capillary conductance by 23±6% and 38±5%, respectively, without changing arteriovenous anastomotic conductance (data not shown).

Human α-CGRP enhanced tissue blood flow dose dependently in extracerebral tissues by 308±49% (30 pmol kg^{-1} min^{-1}) without changing total brain blood flow. In cranial tissues, dural blood flow was increased dose dependently from baseline (4.0±0.8 ml min^{-1} 100 g^{-1}) to a maximum of 367±111% (Figure 7.2). Tissue blood flow was significantly enhanced in all extracranial tissues, including the bones, ears, eyes, fat, muscles, salivary glands, skin and tongue (Figure 7.3). Major increases were observed in the ears and skin, with a maximum increase of 802±198% and 808±160%, respectively (Figure 7.3).

Sumatriptan and dihydroergotamine reversed human α-CGRP-induced increases in extracerebral blood flow by 44±4% and 49±4%, respectively. In tissues, dural blood flow was slightly reduced by both drugs (Figure 7.2). The two drugs partially reversed the increases in blood flow in the ears, skin and muscles, without affecting that to the bones and eyes (Figure 7.3). Additionally, dihydroergotamine reversed blood flow increases in the tongue, salivary glands and fat (Figure 7.3).

Myocardial contractility in vitro

Baseline force of contraction was significantly lower in atrial tissue (50±16 mg, n=6) than in ventricular tissue (595±137 mg, n=10). As shown in Figure 7.4, noradrenaline (10^{-8}-10^{-5} M) increased force of contraction in a concentration-dependent manner in both tissues. With the highest concentration of noradrenaline (10^{-5} M), the force of contraction increased by 163±50 mg (n=6) and 615±106 mg (n=10) in atrial and ventricular trabeculae, respectively. At the end of the protocol, noradrenaline (10^{-5} M) induced positive inotropic responses comparable to the initial responses in ventricular trabeculae (100±6%, n=10) whereas noradrenaline responses in atrial trabeculae were slightly reduced (84±7%, n=6) (Figure 7.4).

In contrast to noradrenaline, human α-CGRP (10^{-9}-10^{-7} M) failed to increase force of contraction in the atrial tissue (baseline: 107±26 mg, 10^{-7} M human α-CGRP: 103±25 mg; n=6 each), but exerted a moderate concentration-dependent positive inotropic effect in ventricular tissue (Figure 7.4). At 10^{-7} M human α-CGRP, ventricular contractile force increased from 545±110 mg at baseline up to 663±120 mg, or 21±3% of the response to 10^{-5} M noradrenaline (n=10).

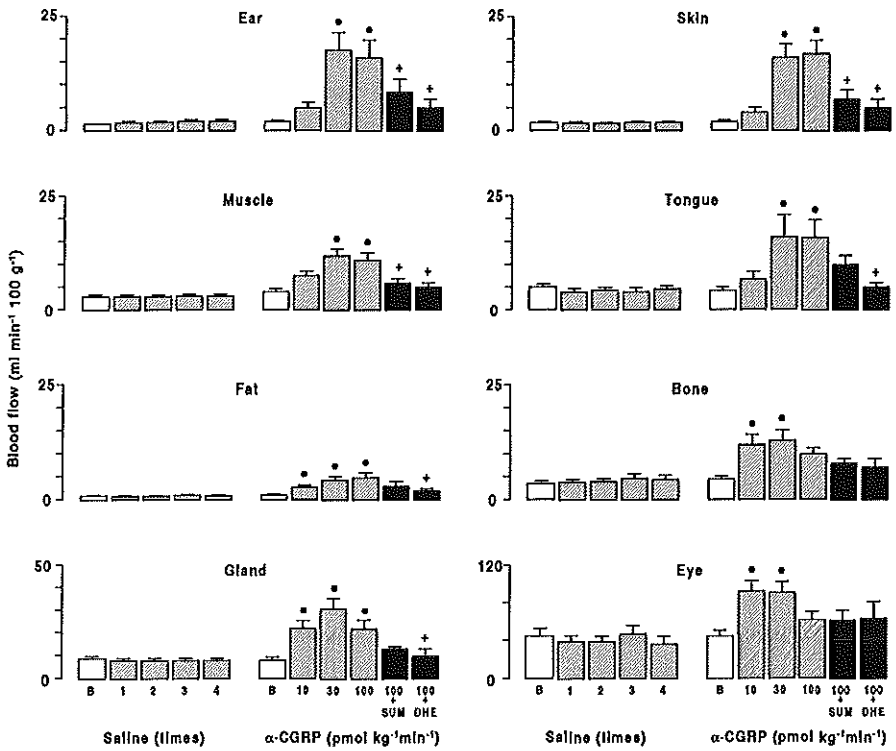

Figure 7.3 Tissue blood flow to extracerebral tissues at baseline (B) and after intracarotid infusions of saline (four times, n=8; left panels) or human α-CGRP (10, 30 and 100 pmol kg^{-1} min^{-1}, n=8; right panels), followed by either sumatriptan (SUM; 300 μg kg^{-1}, i.v.; n=4) or dihydroergotamine (DHE; 100 μg kg^{-1}, i.v.; n=4) in the presence of human α-CGRP (100 pmol kg^{-1} min^{-1}). Data are given as mean ± s.e.mean. *, P≤0.05 versus saline values; +, P≤0.05 versus human α-CGRP.

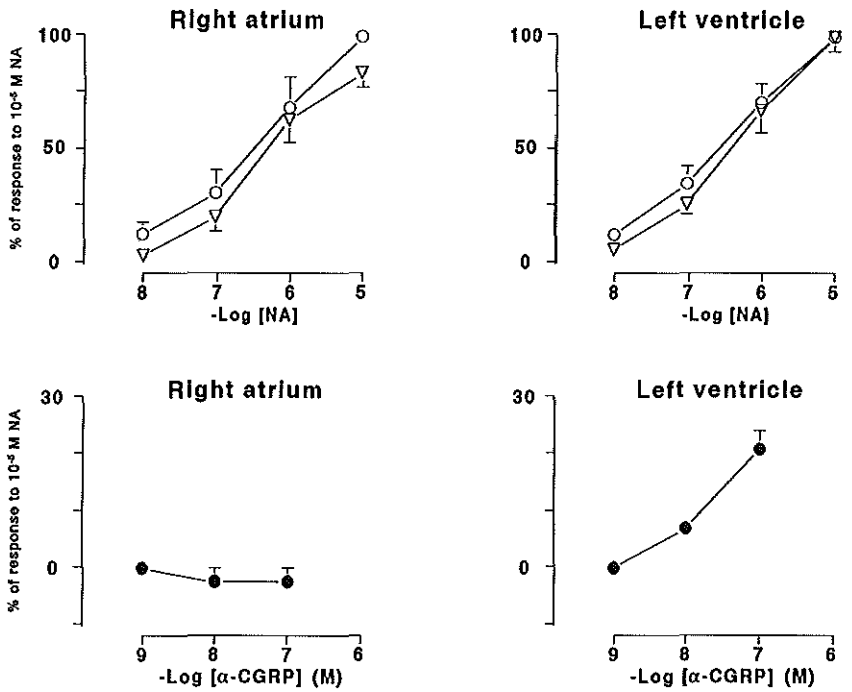

Figure 7.4 Concentration-response curves for noradrenaline (NA; upper panels) (first: ○, second: ▽) and α-CGRP (●; lower panels) in porcine isolated right atrial (n=6) and left ventricular (n=10) trabeculae. Responses are expressed as percentages of the response to NA (10^{-5} M) at baseline. Data are given as mean ± s.e.mean.

7.4 Discussion

Systemic haemodynamics

The systemic haemodynamic effects of human α-CGRP have been described extensively in animals and humans (Franco-Cereceda *et al.*, 1987; Lappe *et al.*, 1987; Gardiner *et al.*, 1989, 1991; Wang *et al.*, 1989). The fall in systemic blood pressure and increase in systemic vascular conductance observed with intracarotid infusions of the two highest concentrations (30 and 100 pmol kg^{-1} min^{-1}) of human α-CGRP are in

accordance with these reports, but also show that the potent vasodilator action of the peptide was not limited to the carotid circulation. Since heart rate increased in parallel with the decline in blood pressure, it is reasonable to assume that changes in heart rate were baroreflex-mediated. Our unpublished observations in two pigs, anaesthetized with pentobarbital and subjected to bilateral vagosympathectomy, support this view; intracarotid infusions of human α-CGRP (up to 100 pmol kg^{-1} min^{-1}) in these animals with markedly inhibited sympathetic tone (see Den Boer *et al.*, 1993) did not increase heart rate, despite a pronounced fall in arterial pressure. It, however, remains possible that in other circumstances (e.g. the use of porcine α-CGRP in pigs) or in other species additional mechanisms, such as a direct chronotropic action or release of catecholamines, may contribute to the observed tachycardia. Indeed, heart rate responses to α-CGRP were attenuated but not completely abolished in dogs after cardiac denervation (Wang *et al.*, 1989). A direct chronotropic effect of rat α-CGRP has also been observed in rat isolated atria (Sigrist *et al.*, 1986), but no tachycardia was detected in conscious rats given human α-CGRP (Gardiner *et al.*, 1989). On the other hand, catecholamine release was reported in dogs and man (Franco-Cereceda *et al.*, 1987; Wang *et al.*, 1989), and ß-adrenoceptor blockade abolished tachycardia in cardiac denervated dogs (Wang *et al.*, 1989).

As previously reported for rats (Lappe *et al.*, 1987), cardiac output increased without changes in stroke volume, most likely, reflecting a reduction in afterload, as indicated by the fall in systemic blood pressure. However, when systemically infused in a comparable dose, human α-CGRP enhanced indices of contractility in conscious rats, despite a marked reduction in stroke index, possibly due to the reduction in venous pressure (Gardiner *et al.*, 1991). If this was also the case in our experiments, the putative positive inotropic action of human α-CGRP (see section *myocardial contractility in vitro*) was masked in pigs by concomitant venodilatation.

Both dihydroergotamine and sumatriptan failed to reverse human α-CGRP-induced systemic haemodynamic changes. Either drug tended to reduce mean arterial blood pressure, significantly so only in the case of sumatriptan. Since the latter was shown previously to reduce cardiac output in anaesthetized pigs (Den Boer *et al.*, 1992b), the small change in this variable may have accounted for the additional reduction in blood pressure. As no significant changes in systemic vascular conductance were observed with either dihydroergotamine or sumatriptan, both drugs exerted a selective vasoconstrictor effect in the carotid vasculature.

Carotid haemodynamics

Carotid vascular pulsations

The increase in carotid vascular pulsations caused by human α-CGRP is partially in accord with previous observations using Doppler flow measurements in humans, when the peptide enhanced pulsatility in the internal carotid artery, but not in the common carotid artery (MacDonald *et al.*, 1989). As argued in chapter 6, these increased pulsations most likely reflect changes in vascular compliance following vasodilatation, though the precise mechanism is unclear. It is to be noted that local pulsatile flow may be influenced by changes in systemic vascular resistance and, furthermore, similar changes in pulsatility are also observed with histamine and nitrovasodilators, precluding the existence of a specific mechanism (Graham & Wolff, 1938; Thomsen *et al.*, 1993; this thesis, chapter 6). The inability of either dihydroergotamine or sumatriptan to change the carotid vascular pulsations induced by human α-CGRP may argue against vasodilatation as the sole underlying mechanism. Additionally, it is possible that the dose of the antimigraine drugs was not sufficient to oppose the enhanced vascular pulsations during continued infusion of a high dose of human α-CGRP.

Carotid blood flow distribution

In keeping with the observations in rats (Gardiner *et al.*, 1989, 1991), human α-CGRP elevated common carotid blood flow dose dependently, associated with pronounced increases in regional tissue blood flow, indicating arteriolar dilatation. In the presence of a high concentration of human α-CGRP, sumatriptan and dihydroergotamine reduced carotid blood flow to an extent similar to that reported in the absence of the peptide (Den Boer *et al.*, 1992a), suggesting that their vasoconstrictor action in this vessel does not depend on the level of circulating human α-CGRP.

As previously reported (Den Boer *et al.*, 1993; this thesis, chapter 6), approximately 21% of the carotid blood flow (compared to over 70% in anaesthetized pigs, Den Boer *et al.*, 1992a) was shunted through arteriovenous anastomoses, indicating that, at baseline, these vessels were largely under sympathetic constrictor tone. Despite its potent vasodilator action, human α-CGRP failed to affect blood flow through arteriovenous anastomoses. These vessels appear to be innervated by sensory nerves (Gorgas *et al.*, 1977; Hales & Molyneux, 1988) and CGRP-containing fibres have been demonstrated immunohistochemically in the proximity of arteriovenous

anastomoses in the dog tongue (Hino *et al.*, 1993). Moreover, in a similar dose, CGRP increased arteriovenous anastomoses blood flow in the sheep hind limb (Mogg *et al.*, 1992). The lack of effect of human α-CGRP in our study is not explained by the difference in peptide used (human α-CGRP versus rat CGRP), since more pronounced vasodilator effects have been reported with human α-CGRP than with rat α-CGRP (Gardiner *et al.*, 1989). As an elevated shunt flow was observed in the sheep hind limb, apparently without significant changes in systemic blood pressure and capillary flow (Mogg *et al.*, 1992), it is possible that the pronounced increase in capillary blood flow in conjunction with the marked rise in systemic vascular conductance prevented the opening of arteriovenous anastomoses in the carotid circulation of the pig by way of a 'steal-like' phenomenon. As nitrovasodilators have been reported to enhance blood flow through arteriovenous anastomoses under comparable conditions (This thesis, chapter 6), it seems unlikely that, in this part of the circulation, nitrovasodilators act via the release of CGRP (Wei *et al.*, 1992; Holzer & Jočic, 1994).

The antimigraine drugs, dihydroergotamine and sumatriptan, have been shown to reduce blood flow through arteriovenous anastomoses in pigs with a high shunt flow (Den Boer *et al.*, 1992a). Since arteriovenous anastomoses were largely closed and repeated infusions with human α-CGRP failed to increase arteriovenous anastomotic blood flow, such a constrictor action could not be detected in the present experiments.

In the porcine carotid circulation, human α-CGRP increased extracerebral blood flow without affecting intracerebral flow. The predominant action on extracerebral tissues, most markedly reflected by the major changes in cutaneous tissues (skin and ears) supports the contention that extracranial vascular dilatation and pulsation indeed have a role in migraine headache (Graham & Wolff, 1938; Drummond & Lance, 1988). However, the absence of cerebral blood flow changes seems to be in conflict with the enhanced cerebral blood flow together with elevated CGRP levels in the external jugular vein following stimulation of the trigeminal ganglion (Goadsby & Edvinsson, 1993) as well as with the increased vessel diameter in the feline cerebral microcirculation observed with CGRP (Wei *et al.*, 1992). This discrepancy is most likely attributable to methodological differences since, in the former study, cerebral blood flow was measured by Laser Doppler flowmetry, detecting changes in large intracerebral vessels (not detected by microspheres), whereas in the latter study CGRP was applied topically, producing relatively high local concentrations. Finally, it may be argued that a putative vasodilator action of human α-CGRP in porcine cerebral capillaries is effectively opposed by cerebral autoregulation.

Within the porcine cranial circulation, dural blood flow was significantly enhanced by human α-CGRP. The dura mater, which is innervated by CGRP-containing nerves (Uddman *et al.*, 1986; Suzuki *et al.*, 1989), is regarded a likely source of headache, and plasma levels of CGRP appear to be elevated in the cranial venous effluent in migraineurs (Friberg *et al.*, 1994). Thus, it is plausible that enhanced dural blood flow, together with increased vascular pulsatility, contributes to migraine headache. However, in spite of an increase in carotid vascular resistance indicating vasoconstriction, antimigraine drugs like dihydroergotamine and sumatriptan failed to reduce dural blood flow in pigs (Den Boer *et al.*, 1992a), whereas sumatriptan reduced feline pial diameter only after topical application (Connor *et al.*, 1992). Moreover, in the present study with presumably high levels of CGRP, both sumatriptan and dihydroergotamine only slightly reduced dural blood flow and failed to reduce carotid vascular pulsations.

Myocardial contractility in vitro

To evaluate the putative positive inotropic action of α-CGRP further, we studied its inotropic responses on isolated trabeculae obtained from right atria and left ventricles from saline treated animals. As both noradrenaline and α-CGRP act via the adenylyl cyclase/cAMP pathway (Brodde *et al.*, 1992; Sigrist *et al.*, 1986; Ishikawa *et al.*, 1987), noradrenaline was used to check the inotropic responsiveness and viability of the tissues, both at the beginning and at the end of the experiments. As in our previous studies (Schoemaker *et al.*, 1992; Du *et al.*, 1994), tissues not responding to noradrenaline at the beginning of the experiment were excluded. All tissues responded to noradrenaline at the end of the experiments, confirming that these tissues remained viable during the course of the experiment.

In contrast to noradrenaline, human α-CGRP failed to increase force of contraction in porcine atrial tissue but increased contractility in ventricular tissue. So far, a positive inotropic action in the atrium rather than ventricles has been observed in the rat (Sigrist *et al.*, 1986; Ishikawa *et al.*, 1987), guinea pig (Saito *et al.*, 1987) and human myocardium (Sigrist *et al.*, 1986; Du *et al.*, 1994), suggesting a rather uniform mode of action, at least on atrial trabeculae. However, the discrepancy may be attributable to species differences. For example, few CGRP specific binding sites have been detected in guinea pig ventricles (Ishikawa *et al.*, 1988), whereas these sites could be demonstrated in porcine ventricular tissue (Miyauchi *et al.*, 1988). At present, no data are available about specific binding sites for CGRP in the porcine atrium.

The ability of human α-CGRP to increase the force of contraction in ventricular trabeculae is in accord with the detection of binding sites for this peptide as well as with the observation of positive inotropic responses in ventricular false tendons (Miyauchi *et al.*, 1988). Moreover, the magnitude of the inotropic response at 10^{-7} M human CGRP, being approximately 25% of the isoprenaline control curve, was comparable with that in our study. Although these moderate ventricular inotropic responses support the hypothesis of a positive inotropic effect in anaesthetized pigs, it remains to be established whether such concentrations actually occur *in vivo*.

In summary and conclusion, intracarotid infusion of human α-CGRP caused arteriolar dilatation in the pig, which was accompanied by hypotension, tachycardia and an increase in cardiac output. Within the carotid circulation, vasodilatation was largely confined to extracerebral tissues and the marked increase in capillary blood flow together with the fall in systemic blood pressure most likely prevented the opening of arteriovenous anastomoses. Furthermore, in the presence of high levels of human α-CGRP, the antimigraine drugs, dihydroergotamine and sumatriptan, reversed blood flow changes induced by human α-CGRP in extracerebral tissues.

Chapter 8

General discussion

A part of the experiments described in this thesis was delineated to study haemodynamic responses and changes in local blood flow induced by inhibition of NO biosynthesis in different animal species. The majority of these effects can be attributed to abolishion of the NO-mediated vasodilator tone, but additional mechanisms like activation of neurohumoral systems may not be excluded. The potential contribution of such mechanisms to the systemic and regional haemodynamic effects of L-NAME is discussed in general.

A dilatation of (extra)cerebral arteriovenous anastomoses has been implicated in the pathophysiology of migraine headache, but the inital vasodilator stimulus is still elusive. Our experiments in the carotid circulation of anaesthetized pigs, a model suitable to study vascular responses to antimigraine drugs, indicate that the endogenous generation of NO may play a role in the regulation of blood flow through these shuntvessels. Furthermore, clinical experimental data seem to imply that changes in NO-mediated pathways occur during migraine headache. The putative relation between NO and the vascular changes during migraine headache is therefore addressed in the second part of this discussion.

8.1 Haemodynamic responses and tissue blood flows

N^G-nitro-L-arginine methyl ester (L-NAME) produced differential haemodynamic responses and changes in tissue blood flow distribution in anaesthetized animals. In the rat, L-NAME exerted a pronounced hypertensive action, independently of the existing vascular tone. In contrast, such a pressor response was absent in cats and pigs, although tissue blood flows were reduced. This species difference is not due to the type of anaesthesia used but may rather be related to differences in compensatory mechanisms, such as differences in baroreflex sensitivity and differences in regional production of NO. This is illustrated by the finding that in the intact rat, pressor responses to

L-NAME are partially compensated by reflex bradycardia whereas in the pig the expected rise in blood pressure is opposed by a fall in cardiac output probably due to profound pulmonary vasoconstriction.

Our experiments with L-NAME in anaesthetized animals support the general contention that NO is released at a basal rate and, accordingly, plays a role in the regulation of local blood flow and blood pressure. Nevertheless, both haemodynamic responses and changes in local blood flow distribution following L-NAME were attenuated, but not always reversed by the endogenous substrate L-arginine. This may suggest that other, NO-independent, mechanisms were involved in the effects of L-NAME. Buxton and coworkers (1993) claimed that the L-NAME-induced responses are explained by its atropine-like activity (Buxton *et al.*, 1993). However, L-NAME did not change the magnitude of the hypotensive responses to acetylcholine in rats and cats (see chapter 2) and L-NAME is rapidly dealkylated *in vivo* to L-NNA (Schwarzacher & Raberger, 1992), which is devoid of antimuscarinic actions (Buxton *et al.*, 1993). Moreover, Cheng and colleagues (1994) demonstrated that the bradycardic response upon efferent vagal stimulation, a muscarinic receptor-mediated reponse not dependent on the release of NO, was not impaired by a high dose of L-NAME but abolished by atropine. Therefore, it is unlikely that the effects of L-NAME described in this thesis were mediated through blockade of muscarinic receptors. The apparent inability of L-arginine to reverse the actions of L-NAME is probably best explained by the high affinity of L-NAME for the enzyme NOS, requiering a high concentration of substrate to compete for the active site of the enzyme.

Although the haemodynamic effects and changes in regional blood flow during inhibition of endothelial NO production in intact anaesthetized animals can be attributed to abolishion of the NO-mediated vasodilator tone, it is unclear which vasoconstrictor stimulus served as the functional counterpart and, hence, accounts for the rise in peripheral resistance and blood pressure. Since vasoconstriction is largely dependent on the level of sympathetic tone under normotensive conditions and vasoconstrictor responses to adrenergic agonists are potentiated by NO-inhibitors, it is likely that in the abscence of NO formation vasoconstriction mediated by circulating catecholamines may dominate. In addition, central sympathoexcitatory effects have been proposed to participate in the hypertensive action of NO-synthesis inhibitors. Sakuma *et al.* (1992) demonstrated that L-NMMA causes a long-lasting increase in renal sympathetic nerve activity, susceptible to sympathectomy. Enhanced renal

sympathetic activity and hypertensive responses were also observed with micro-injections of L-NMMA into the nucleus tractus solitarius of intact, and sinoaortic denervated and vagotomized animals (Harada *et al.*, 1993). However, Halbrügge and colleagues (1991) reported that the L-NMMA-induced pressor effect in anaesthetized animals is accompanied by a reduction, rather than an increase in catecholamine release. Furthermore, the pressor response of L-NAME in the rat has been shown to be resistant to ganglionic blockade and blockade of adrenergic receptors (Wang & Pang, 1991; Chuy *et al.*, 1992). Moreover, our experiments in the pithed rat preparation suggest that the hypertensive action of L-NAME does not primairely depend on the integrety of the sympathetic nervous system. Thus, it is conceivable that activation of the central sympathetic system is of minor importance in our experiments.

Likewise, the influence of the renin-angiotensin system on the systemic pressor responses in anaesthetized animals is unclear. Neither converting enzyme inhibition nor angiotensin II receptor antagonism reduced the hypertensive effects of NO synthesis inhibition in the rat (Wang & Pang, 1991; Zambetis *et al.*, 1991; Sigmon & Beierwaltes, 1994). Nevertheless, the angiotensin II receptor antagonist losartan attenuatted the L-NAME-induced reductions in tissue blood flows and the increase in peripheral resistance in anaesthetized rats (Sigmon & Beierwaltes, 1994). Furthermore, while comparing the interaction of L-NAME and angiotensin II receptor blockade in conscious and anaesthetized rats, they noted that the ability of losartan to oppose regional haemodynamic responses to L-NAME is more obvious in the anaesthetized rat than in the conscious rat and proposed that the vasoconstrictor action of angiotensin II is the major antagonist of NO-mediated vasodilation when the activity of the renin-angiotensin system and the level of circulating angiotensin II are elavated. If this hypothesis is correct, it is conceivable that angiotensin II may also account for the haemodynamic responses observed in the pithed rat preparation, since in this model peripheral vascular resistance and blood pressure is maintenained by a high level of endogenous angiotensin II (De Jonge *et al.*, 1982).

Our experiments in rats and cats demonstrated that the hypotensive response to both acetylcholine and serotonin are mediated largely independent of the generation of NO by the vascular endothelium, suggesting a stricking dissimilarity between in vitro and in vivo experiments. In case of acetylcholine-evoked hypotension, evidence is currently accumulating that apart from its well-kown ability to stimulate vascular NO production, acetylcholine produces vasorelaxation via endothelium-dependent smooth muscle hyperpolarization. The contribution of smooth muscle hyperpolarization to

vascular relaxation seems inversely related to the vessel diameter. Accordingly, relaxations to acetylcholine in small resistance vessels are mediated via the release of EDHF with only a minor contribution of NO (Adeagbo *et al.*, 1993; Garland *et al.*, 1995). Interestingly, Kilpatrick & Cocks (1994) demonstrated in the porcine coronary artery that when NO-synthesis is blocked the relaxant response to endothelium-dependent vasodilators is preserved by hyperpolarization, indicating that the release of EDHF and smooth muscle hyperpolarization may serve as a backup system to control arterial diameter.

8.2 Nitric oxide and migraine

Inspite of an ongoing debate regarding its relevance as a cause of migraine headache, (extra)cranial vasodilatation and arteriovenous anastomoses shunting has been reported during the headache phase and the therapeutic effect of serotonergic agonists has been related to selective vasoconstriction in the carotid circulation. The trigger for local vasodilation is yet unclear and a number of potential candidates have been proposed. According to the neurogenic inflammation theory of Moskowitz (1992), neuropeptide release from perivascular nerves of the trigeminal system could account for the local vascular changes in migraine. These neuropeptides, substance P and CGRP, are indeed potent vasodilators but they do not cause headache when injected in humans, and our experiments with intracarotid infusions with CGRP in pigs failed to detect significant changes in carotid arteriovenous anastomotic blood flow, indicating that this peptide may not be the principal mediator of vascular changes in migraine. The observation that inhibition of NOS in cats induced a reduction of the microsphere content of the lungs, a measure for blood flow through systemic arteriovenous anastomoses, led to the hypothesis that NO contributes to the regulation of tone in these blood vessel segments and hence may be involved in the vascular events of migraine. This concept was evaluated in detail in anaesthetized pigs, demonstrating that L-NAME reduced blood flow through systemic arteriovenous anastomoses. Intracarotid administration of L-NAME reduced blood flow in the carotid artery, which was accompanied by a reduction of flow through extracerebral anastomoses. Since arteriovenous anastomotic conductance was restored by L-arginine a NO-dependent mechanism is likely. Subsequent experiments using NO donors showed that under low anastomotic blood flow conditions NO may enhance blood flow in the carotid

vasculature including arteriovenous anastomoses. Together, the results from our experiments suggest that arteriovenous anastomoses in this part of the circulation, at least in pigs, are indeed under NO-mediated vasodilator tone. Recent findings by Van Es *et al.* (1995), suggest that our results may be applicable to humans. These authors reported that the NO-donor sodium nitroprusside increases finger blood flow, which is predominantly determined by flow through arteriovenous anastomoses.

Additional support for the contention that NO is involved in the vascular changes of migraine is provided by observations from experimental headache. Various vasodilator drugs, including nitrates and nitrites, cause headache as a side effect. For this reason, nitrovasodilators have been used to provoke experimental headache in man. In healthy volunteers, isosorbide mononitrite caused a dose-related headache, which resembled migraine in some but not all subjects. The headache was bilateral and pulsating of nature, and was aggravated by physical activity. Furthermore, headache was accompanied by a dose-dependent dilatation of the superficial temporal artery (Iversen *et al.*, 1992). Secondly, glyceryl trinitrate-induced headache was augmented by pretreatment with N-acetylcysteine, a donor of sulphydryl-groups which may facilitate the liberation of NO from vasodilators. The potentiating effect on headache was paralleled by enhanced dilatory responses in the temporal artery (Iversen, 1992).

Repeating these experiments in migraine patients, Olesen reported that infusion of glyceryl trinitrate induced a more intense dose-dependent headache and middle cerebral artery dilatations, than in non-migraineurs (Olesen *et al.*, 1993, Thomsen *et al.*, 1993). Moreover, some of the migraineurs experienced a second migraine-like headache within 24 h, suggesting a delayed release of other mediators.

As with nitrovasodilators, administration of histamine to migraine patients caused headache which was paralleled by vasodilatation and enhanced vascular pulsations on the side of the pain (Graham & Wolff, 1938). Although the histamine-induced headache was shown to be clinically distinct from migraine headache, headache was experienced by migraine patients rather than by control subjects. Subsequent injection of the H_1 receptor antagonist mepyramine abolished histamine-induced headache responses (Krabbe & Olesen, 1980). Histamine is known to cause relaxations in isolated cranial blood vessels, most likely via endothelial H_1 receptors to release endogenous NO, since relaxations are attenuated by L-NMMA and abolished by endothelial denudation (Toda, 1990; Fujiwara *et al.*, 1992). These findings have led Olesen *et al.* (1993) to conclude that migraineurs are more sensitive to both exogenous and endogenous NO.

Similar to migraine, attacks of cluster headache are provoked by administration of glyceryl trinitrate (Drummond & Anthony, 1985; Dahl *et al.*, 1990). These attacks are also accompanied by an increase in temporal artery pulse amplitude on the side of the headache, and both headache intensity and temporal pulsations are reduced by breathing pure oxygen (Drummond & Anthony, 1985). Furthermore, in both types of headache, concentrations of plasma CGRP appear to be elevated during the attack (Goadsby *et al.*, 1990; Goadsby & Edvinsson, 1994). Topical application of nitrovasodilators was shown to stimulate the release of CGRP from perivascular sensory nerve endings in pial arteries as well as cutaneous microvessels (Wei *et al.*, 1992; Holzer & Jocic, 1994). If this is also the case in humans, the rise in plasma CGRP may be the consequence of increased sensitivity or activity of NO-mediated pathways. Consistent with this view, oxygen was shown to alleviate pain and to normalize elevated levels of plasma CGRP in patients suffering from cluster headache (Goadsby & Edvinsson, 1994). In this respect, it is interesting to note that high oxygen tension facilitates the inactivation of NO and may limit its effect.

Putative mechanisms

Since enhanced sensitivity is observed in migraine patients, both following endothelium dependent (histamine) and independent (nitrovasodilators) stimuli, this effect is likely due to changes downstream of the endothelium. One possible explanation is provided by alterations at the level of the second messenger system, such as upregulation of the guanylyl cyclase system (Olesen *et al.*, 1993). On the other hand, Fozard and Kalkman (1994) reasoned that the release of 5-HT in the early stages of the attack may activate endothelial 5-$HT_{2B/2C}$ receptors and enhanced sensitivity of these receptors could explain the role of NO in migraine. Changes in the metabolism and distribution of 5-HT as well as its pharmacology have indeed been related to pathophysiology of migraine (see Lance, 1993; Saxena, 1995). Furthermore, 5-HT has been shown to induce endothelium-dependent responses in isolated porcine peripheral arteries with pharmacological similarities to the 5-HT_{2C} receptor (Sumner, 1991; Glusa & Richter, 1993). However, we were unable to detect a NO-dependent vasodilator response for 5-HT in pithed rats and, moreover, the presence of this type of receptor on (extra)cranial arteries and arteriovenous anastomoses has not yet been shown.

Nitroxidergic nerves

The vascular events in migraine are not easily explained by simple changes in basal release of NO from the vascular endothelium and, hence, additional NO-mediated pathways may be involved. As noted in chapter 5, L-NAME did not reduce cerebral blood flow in pigs and high systemic doses or topical application of NOS-inhibitors are requiered to reduce cerebral blood flow (Faraci, 1991; Kovách *et al.*, 1992; Prado *et al.*, 1992) indicating an action distinct from the endothelium. Within the brain NO, derived from neurons and astrocytes, is believed to couple local blood flow to neural activity (Ideacola, 1993). In addition, NO may transmit information from non-adrenergic and non-cholinergic nerves to (extra)cranial arteries. Both NADPH-diaphorase histochemistry and immunocytochemical studies using antibodies against NOS have identified dense NOS-containing perivascular nerves innervating the circle of Willis, large cerebral, pial and temporal arteries in several species (Estrada *et al.*, 1993; Iadecola *et al.*, 1993; Nozaki *et al.*, 1993; Yoshida *et al.*, 1993). In humans, immunoreactive fibres were observed within the central nervous system (Springall *et al.*, 1992), but only sparse immunoreactivity was detected in cerebral vessels, presumably due to the use of post-mortem material (Nozaki *et al.*, 1993). Nitroxidergic nerves appear to originate from the sphenopalatine ganglion, since immunoreactivity was abolished either by efferent nerve section or lesions in this parasympathetic ganglion (Nozaki *et al.*, 1993; Yoshida *et al.* 1993). A portion of NOS-positive neurons is also immunoreactive to vasoactive intestinal polypeptide (VIP), suggesting that NO and VIP may be released from the same nerve terminals (Nozaki *et al.*, 1993).

The putative functional role of nitroxidergic nerves in the regulation of local blood flow is suggested by experiments performed on isolated cerebral and extracerebral blood vessels. That is, relaxations of the dog superficial temporal artery upon transmural nerve stimulation are associated with an increase in tissue cGMP-level and are effectively antagonized by inhibition of NOS and ganglion blockade (Okamura *et al.*, 1993). NOS-inhibition and destruction of the sphenopalatine ganglion also prevented relaxant responses of canine and porcine cerebral and temporal arteries induced by the ganglionstimulant nicotine as well as by transmural nerve stimulation (Chen & Lee, 1993; Toda *et al.*, 1993).

At present, evidence for a physiological role in vivo is limited; stimulation of the sphenopalatine ganglion in rats increases cortical blood flow, which is attenuated by L-NAME (Morita-Tsuzuki *et al.*, 1993). Furthermore, heat-induced vasodilatation,

sensitive to local nerve block, is reduced by L-NNA in rabbits (Taylor & Bishop, 1993). However, animal studies have also shown that stimulation of trigeminal fibers increases blood flow in the carotid circulation together with a rise in skin temparature. These changes in carotid blood flow seem to occur, at least in part, via a para-sympathetic reflex involving the seventh cranial nerve and the sphenopalatine ganglion (Lambert *et al.*, 1984). So far, this reflex vasodilatation has been ascribed predominantly to the release of VIP and CGRP from perivascular nerves (Goadsby & Macdonald, 1985; Beattie & Connor, 1994), but in view of the co-localization of VIP- and NOS-immunoreactivity a nitroxidergic vasodilatory component may be considered. If this reflex axis is also operative in migraine headache, it is reasonable to assume a contribution of nitroxidergic nerves to the local vascular events in migraine.

The role of these nitroxidergic nerves in the pathophysiology of migraine is yet to be defined. In order to discreminate between endothelium-dependent vascular effects and neuronal evoked responses the development of selective type I NOS-inhibitors is a prerequisite for future experiments. This may eventually lead to a novel therapeutic approach in the treatment of migraine. However, it is also to be expected that type I selective NOS-inhibitors may cause major side effects since this isoform is abundantly present within the central nervous system.

In conclusion, NO contributes differentially to the regulation of local vascular tone and, hence, regional blood flow in anaesthetized animals. The ability of endogenous NO to change blood flow through carotid arteriovenous anastomoses seems relevant for migraine headache. This may lead to the development of novel antimigraine therapy.

Appendix

Summary

The vascular endothelium has been recognized as an important regulatory organ with multiple physiological functions. Endothelial cells may contribute to the cardiovascular homeostasis via the release of vasoactive substances with opposite action, which may influence tone of the underlying smooth muscle and modulate local blood flow. The potent vasodilator property of NO has led to the view that alterations in the physiology of this molecule may be important under pathophysiological conditions and clinical experimental data seem to suggest that NO is involved in the aetiology of migraine.

In *chapter 1*, the clinical features of the migraine syndrome and the vascular changes during migraine attacks are discussed. Vasodilatation of large extracranial, basal cerebral blood vessels and, arteriovenous anastomoses can be observed during the headache phase of migraine. Local vasodilatation is often accompanied by increased vascular pulsatility and the ensuing pain may be due to a depolarization of perivascular sensory nerves around these arteries. In addition, local release of vasoactive neuropeptides, like calcitonin gene-related peptide (CGRP), may maintain the vascular changes during the attack.

The second part of this chapter describes the discovery, chemical identity and origin of endothelium-derived relaxing factor (EDRF), and provides an overview of the current knowledge of the various endothelium-derived vasoactive factors. The factors mediating vasocontriction include thromboxane and prostaglandin H_2, the endothelins, and angiotensin II. Local vascular tone may be reduced via the release of prostacyclin and EDRF. In addition, smooth muscle hyperpolarization, attributed to the endothelial release of an unidentified hyperpolarizing factor (EDHF), may also participate in vasorelaxation.

In *chapter 2*, the haemodynamic effects of the NOS-inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) and its potential ability to attenuate hypotensive responses to the endothelium-dependent vasodilator acetylcholine were investigated in anaesthetized rats and cats. L-NAME caused a sustained hypertensive response and a moderate

bradycardia in rats. Hypertension was partially reversed by the endogenous substrate L-arginine. In contrast, L-NAME failed to modify systemic haemodynamic parameters in cats. Subsequent administration of L-arginine did not exert any significant haemodynamic effect in cats treated with L-NAME, but caused a moderate reduction in heart rate and increases in cardiac output and stroke volume in saline-treated control cats. In the cat, L-NAME reduced tissue blood flows in several abdominal organs. Administration of L-arginine produced a significant increase in blood flow in the lungs, mesentery, kidneys and liver, which was not observed in cats previously treated with L-NAME. The magnitude of the acetylcholine-induced hypotensive response, was unaffected by L-NAME. However, the duration of these responses was reduced by L-NAME in both rats and cats.

The results from these experiments reveal a marked difference between the haemodynamic effects of L-NAME in rats and cats. They suggest that in cats, unlike rats, the contribution of L-arginine-derived NO to the regulation of blood pressure is rather limited, although such a pathway may exist in various tissues. Furthermore, the hypotensive response to acetylcholine in both species seems to be mediated largely via NO-independent pathways.

In *chapter 3*, the haemodynamic effects of L-NAME were studied in rats with low vascular tone. In the pithed rat preparation, L-NAME elavated mean arterial blood pressure to a similar extent as previously observed in anaesthetized rats, without changing heart rate. Blood pressure responses to L-NAME were unaffected by the α_1 adrenoceptor and 5-HT_2 receptor antagonist ketanserin.

As relaxant responses of isolated vascular preparations to 5-hydroxytryptamine (5-HT) were shown to dependent partly on functional endothelium, we evaluated the possible involvement of endothelial NO-release in blood pressure responses to 5-hydroxytryptamine (5-HT). The hypotensive responses to 5-HT in the presence of ketanserin were augmented by L-NAME as well by the α_1-adrenergic agonist phenylephrine. L-NAME also tended to prolong the duration of the response to 5-HT. The magnitude of hypertensive responses to 5-HT was unaffected by L-NAME, but L-NAME extended the duration of these responses.

These experiments suggest that L-NAME increases blood pressure in animals with a low vascular tone, probably by inhibiting the basal release of NO. However, the

hypotensive responses to 5-HT seem to be largely independent of NO-release by the endothelium, but the hypertensive responses to 5-HT appear to be limited by concommitant release of NO in the pithed rat.

In *chapter 4*, haemodynamic responses evoked by L-NAME were analyzed in more detail in the anaesthetized pig. Tissues containing arteriovenous anastomoses were identified and the effects of NOS-inhibition on total systemic and regional arteriovenous shunting analyzed.

L-NAME reduced systemic and pulmonary artery conductance and cardiac output, but heart rate and mean arterial blood pressure remained unchanged. L-arginine reversed the systemic and pulmonary haemodynamic changes induced by L-NAME.

The difference between blood flows simultaneously measured with 15 and 50 μm microspheres, which can be equated to blood flow through arteriovenous anastomoses with a diameter between about 28 and 90 μm, was reduced by L-NAME.

These results suggest that in the anaesthetized pig (i) NO is involved in the regulation of both systemic and pulmonary vascular conductance, (ii) the decrease in systemic vascular conductance is in part due to constriction of systemic arteriovenous anastomoses, and (iii) the decrease in pulmonary vascular conductance, leading to reduction of cardiac output.

The role of NO biosynthesis in the regulation of blood flow through carotid arteriovenous anastomoses of the anaesthetized pig is described in *chapter 5*. Apart from the highest dose, L-NAME caused no major changes in the systemic haemodynamic variables, whereas L-arginine slightly reduced mean arterial blood pressure in control animals.

L-NAME caused a dose-dependent decline in common carotid artery blood flow and conductance, which was entirely confined to the arteriovenous anastomotic part of the carotid circulation. Subsequent intracarotid injection of L-arginine reversed the reduction in total carotid conductance almost completely and that in the arteriovenous anastomotic part partially.

These experiments indicate that the L-arginine-NO pathway contributes little to the regulation of tissue perfusion in the porcine carotid circulation. In contrast, NO seems to play an important role in shunting arterial blood through arteriovenous anastomoses in the anaesthetized pig.

In *chapter 6*, the putative role of nitric oxide (NO) in the regulation of capillary and arteriovenous anastomotic blood flow was investigated in the carotid circulation of the anaesthetized pig. For this purpose, the effects of intracarotid infusions of two NO donors, nitroprusside sodium (NPR) and S-nitroso-N-acetylpenicillamine (SNAP) were studied on systemic haemodynamics, and carotid blood flow as well as its distribution.

Apart from heart rate, which increased after both NPR and SNAP, no major changes in systemic haemodynamic variables were observed. Both NPR and SNAP increased common carotid blood flow, vascular conductance and vascular pulsations dose-dependently. Both NPR and SNAP enhanced total capillary blood flow and conductance. In contrast to NPR, arteriovenous anastomotic blood flow and conductance were increased by SNAP. At the tissue level, capillary blood flow increases following NPR or SNAP were reflected by an increase in both extracerebral and dural blood flow without changes in total brain blood flow.

These results indicate that both NO donors cause arteriolar dilatation together with enhanced vascular pulsations in the carotid circulation of the pig. The pronounced arteriolar dilatation limits the effect of NO donors on arteriovenous anastomoses, probably by way of a 'steal' phenomenon. Together, the findings described in this chapter support the contention that dilatation of intra- and extracranial arteries and arteriovenous anastomoses leads to increased vascular pulsations, which (rather than increased blood flow *per se*) could, at least in part, be responsible for the headache caused by nitrovasodilators.

Chapter 7 describes the effects of the sensory neuropeptide, calcitonin gene-related peptide (α-CGRP) on haemodynamics and carotid blood flow distribution in the anaesthetized pig. The peptide has been implicated in the pathogenesis of migraine headache and its release, in some vessel segments, is apparently initiated by NO. Human α-CGRP increased carotid artery blood flow and conductance dose-dependently, together with an enhancement in vascular pulsations. These effects were associated with a fall in systemic blood pressure with concomitant increases in heart rate and cardiac output. The increase in carotid blood flow was reflected by an increase in total capillary blood flow, predominantly to extracerebral tissues including the dura, whereas blood flow through arteriovenous anastomoses remained stable. The antimigraine drugs, dihydroergotamine and sumatriptan, reduced carotid blood flow and its capillary fraction without affecting systemic vascular conductance. These drugs

reversed blood flow increases due to human α-CGRP in most extracerebral tissues, but failed to reduce dural blood flow.

These data indicate that human α-CGRP caused arteriolar dilatation together with a fall in blood pressure in the pig. The fall in systemic arterial blood pressure and the marked increase in capillary blood flow most likely negated the opening of arteriovenous anastomoses. Furthermore, the antimigraine drugs, dihydroergotamine and sumatriptan, were able to reverse blood flow changes induced by human α-CGRP in the porcine carotid circulation.

In *chapter 8*, the experimental results of this thesis are addressed in general and, in addition, the putative role for NO in the pathophysiology of migraine is discussed. The majority of haemodynamic and local blood flow responses to L-NAME can be attributed to a reduction of the basal NO-mediated vasodilator tone rather than to its proposed antimuscarinic action. The functional counterpart of NO responsible for the rise in systemic and local vascular resistance seems to be provided, at least in intact animals, by circulating catecholamines.

The initial stimulus causing vasodilatation during migraine is largely unknown and locally released CGRP may not be the principal mediator. Although this neuropeptide is a potent vasodilator, it causes no headache in humans and did not induce arteriovenous anastomotic shunting in the pig. In contrast, our experiments in the pig indicate that arteriovenous anastomotic tone can be regulated by endothelium-derived NO and clinical experimental studies suggest an upregulation of NO-mediated pathways in migraine patients. Nitrovasodilators evoke migraine-like headache, which is accompanied by dilatation of extracerebral and basal cerebral arteries. Both headache and vasodilatation is more pronounced in migraine patients than in healthy subjects. Similarly, headache and local vasodilatation has been observed in migraine patients with the endothelium-dependent vasodilator histamine. Since an enhanced sensitivity is observed in migraine patients following endothelium-dependent and independent generation of NO, this phenomenon is not easily explained by simple changes in basal NO-release from the vascular endothelium. A putative contribution of nitroxidergic nerves to the regulation of cranial blood flow is proposed.

In conclusion, NO contributes differentially to the regulation of local vascular tone and, hence, regional blood flow in various species. The ability of endogenous NO to change blood flow through carotid arteriovenous anastomoses seems relevant for migraine headache. This may lead to the development of novel antimigraine therapy.

Samenvatting

Het vasculaire endotheel vormt een belangrijk regulerend orgaan dat een verscheidenheid aan fysiologische functies vervult. De endotheelcellen dragen bij aan het handhaven van de cardiovasculaire homeostasis door de afgifte van vasoactieve factoren met tegengestelde werking, welke de tonus van de onderliggende gladde spierlaag kunnen veranderen en de bloedstroom lokaal kunnen beinvloeden. Uit de potente vasodilatoire werking van stikstof oxide (NO) valt af te leiden dat veranderingen in de fysiologische werking van deze verbinding vooral van belang kan zijn onder pathofysiologische omstandigheden en klinisch experimenteel onderzoek duidt op een mogelijke rol voor NO in de aetiologie van migraine.

In *hoofdstuk 1* worden de klinische symptomen van het migraine syndroom en de vasculaire veranderingen tijdens een migraine aanval besproken. De hoofdpijn fase van een migraine aanval gaat gepaard met een verwijding van grote extracraniële en basale cerebrale arteriën. Bovendien is verwijding van arterioveneuze anastomosen beschreven. De lokale vaatverwijding gaat veelal samen met versterkte vasculaire pulsaties en de hoofdpijn kan worden veroorzaakt door een depolarisatie van perivasculaire sensore zenuwen. Een lokale vrijzetting van vasoactieve neuropeptiden, zoals calcitonin gene-related peptide (CGRP), kan bijdragen aan het onderhouden van de vasculaire veranderingen tijdens de hoofdpijn aanval.

Het tweede gedeelte van dit hoofdstuk beschrijft de ontdekking, de chemische karakterisering en de oorsprong van de endotheel-afhankelijke relaxerende factor (EDRF), en geeft een overzicht van de huidige kennis van de verschillende endotheel-afhankelijke vasoactieve factoren. De factoren die lokale vasoconstrictie veroorzaken zijn thromboxaan en prostaglandine H_2, de endothelines en het angiotensine II. Lokale verlaging van de vaattonus wordt gemedieerd via de afgifte van prostacycline en EDRF. Daarnaast draagt hyperpolarisatie van de gladde spiercel, via een door het endotheel gegenereerde onbekende hyperpolariserende factor (EDHF), bij aan de vaatverwijding.

In *hoofdstuk 2* worden de hemodynamische effecten van de NO-syntheseremmer N^G-nitro-L-arginine methyl ester (L-NAME) en de mogelijkheid met deze verbinding de hypotensieve respons, veroorzaakt door de endotheel-afhankelijke vasodilator acetylcholine, te remmen beschreven in genarcotiseerde ratten en katten.

L-NAME induceerde een langdurig hypertensief effect en een bescheiden verlaging van de hartfrequentie in ratten. Het hypertensieve effect werd tegengegaan door het endogene substraat L-arginine. L-NAME vertoonde echter geen hemodynamische respons in katten. Toediening van L-arginine veroorzaakte geen significante hemodynamische effecten in katten welke eerder behandeld waren met L-NAME. Daarentegen induceerde L-arginine een geringe verlaging van de hartfrequentie, een toename van het hartminuutvolume en slagvolume in katten behandeld met fysiologisch zout.

In de kat verlaagde L-NAME de bloedstroom naar verschillende abdominale organen. Toediening van L-arginine resulteerde in een significante toename van de bloedstroom naar de longen, het mesenterium, de lever en nieren. Deze respons was minder in dieren behandeld met L-NAME. De grootte van de hypotensieve respons geinduceerd door acetylcholine werd door L-NAME niet beinvloed, maar de duur van deze respons werd door L-NAME bekort in zowel de rat als de kat.

De resultaten van deze experimenten tonen een uitgesproken verschil in de hemodynamische effecten van L-NAME tussen de rat en de kat. De bijdrage van NO aan de regulatie van de bloeddruk lijkt, in tegenstelling tot de rat, beperkt in de kat. Een regulatoire werking voor NO lijkt echter wel te bestaan in verschillende weefsels. In beide diersoorten lijkt de hypotensieve werking van acetylcholine te worden gemedieerd via een NO-onafhankelijk mechanisme.

In *hoofdstuk 3* worden de hemodynamische effecten van L-NAME in ratten met een lage vasculaire tonus beschreven. In de gepende rat verhoogde L-NAME de gemiddelde bloeddruk in een vergelijkbare mate als in de intacte genarcotiseerde rat. De verlaging van de hartfrequentie bleef echter achterwege. De bloeddruk effecten van L-NAME werden niet beinvloed door voorbehandeling met de α_1 adrenerge en $5HT_2$ serotonerge receptor antagonist ketanserine.

Aangezien de vasorelaxerende werking van serotonine in geisoleerde vasculaire preparaten afhankelijk lijkt te zijn van functioneel endotheel, werd de mogelijke bijdrage van NO-vrijzetting door het endotheel aan de bloeddruk effecten van 5-hydroxytryptamine (5-HT) eveneens onderzocht. In de aanwezigheid van ketanserine werden de hypotensieve responsen van 5-HT versterkt door zowel L-NAME als de α_1-adrenerge agonist fenylefrine. De duur van de bloeddrukdaling werd enigszins verlengd door L-NAME. De grootte van de hypertensieve respons van 5-HT werd door L-NAME niet beinvloed, maar de duur van deze respons werd verlengd.

Deze resultaten suggereren dat L-NAME de bloeddruk verhoogt in dieren met een lage vaattonus, waarschijnlijk via remming van de basale afgifte van NO. De hypotensieve effecten van 5-HT zijn daarentegen onafhankelijk van NO-vrijzetting door het endotheel. In de gepende rat lijken de hypertensieve responsen van 5-HT te worden tegengegaan door een gelijktijdige vrijzetting van NO.

In de, in *hoofdstuk 4*, beschreven experimenten werden de hemodynamische effecten van L-NAME nader geanalyzeerd in het genarcotiseerde varken. Bovendien werd het effect van de remming van het enzym NOS op de totale systemische- en regionale bloedstroom door arterioveneuze anastomosen bestudeerd.

L-NAME verlaagde de systemische- en pulmonale vasculaire conductantie en het hartminuutvolume, zonder veranderingen in hartfrequentie en gemiddelde arteriële bloeddruk. De systemische en pulmonale hemodynamische veranderingen werden tegengaan door L-arginine.

Het verschil in bloedstroom, gemeten via simultane injecties met microsferen met een diameter van respectieveljk 15 μm en 50 μm, werd door L-NAME gereduceerd, hetgeen kan duiden op een afname van de bloedstroom door arterioveneuze anastomosen met een diameter tussen 28 en 90 μm.

Uit deze resultaten mag worden afgeleid dat in het genarcotiseerde varken (i) NO betrokken is bij de regulatie van zowel de systemische als de pulmonale vasculaire conductantie, (ii) de afname van de systemische vasculaire geleidbaarheid deels veroorzaakt wordt door een constrictie van systemische arterioveneuze anastomosen en, (iii) door een afname van de pulmonale vasculaire conductantie, resulterend in een daling van het hartminuutvolume.

In *hoofdstuk 5*, werd de bijdrage van de biosynthese van NO aan de regulatie van de bloedstroom door arterioveneuze anastomosen onderzocht in het vaatbed van de arteria carotis van het genarcotiseerde varken.

Behalve in de hoogste dosering, had L-NAME geen invloed op de systemische hemodynamische variabelen, terwijl L-arginine de gemiddelde arteriële bloeddruk enigszins verlaagde. Remming van de NO-synthese resulteerde in een dosis-afhankelijke afname van de bloedstroom door de arteria carotis en de conductantie van het carotide vaatbed. De afname beperkte zich geheel tot het arterioveneuze gedeelte van deze circulatie. Toediening van L-arginine herstelde de vasculaire conductantie van

de arteria carotis vrijwel geheel en de conductantie van de arterioveneuze fractie gedeeltelijk.

Deze experimenten duiden erop dat de vorming van NO uit L-arginine weinig bijdraagt aan de regulatie van de weefseldoorbloeding in het vaatbed van de arteria carotis van het varken. Daarentegen lijkt NO een belangrijke invloed te hebben op de afvoer van bloed door arterioveneuze anastomosen in het genarcotiseerde varken.

Hoofdstuk 6 beschrijft de effecten van de NO-donoren nitroprusside natrium (NPR) en S-nitroso-N-acetylpenicillamine (SNAP) op de capillaire en arterioveneuze anastomotische bloedstroom in de carotide circulatie van het varken.

Met uitzondering van de hartfrequentie, veroorzaakte intracarotide infusie met deze nitrovasodilatoren geen systemische hemodynamische veranderingen. De bloedstroom door de arteria carotis communis, de vasculaire conductantie, en de pulsaties van deze arterie werden zowel door NPR als SNAP dosis-afhankelijk verhoogd. Beide NO-donoren induceerde een toename van de totale capillaire bloedstroom en conductantie. In tegenstelling tot NPR, werd de bloedstroom door arterioveneuze anastomosen en de conductantie in dit deel van de carotide circulatie verhoogd door SNAP. De toename van de totale capillaire doorbloeding werd weerspiegeld door een toename van de bloedstroom in zowel extracerebrale weefsels als de dura, zonder noemenswaardige veranderingen in de overige intracraniële weefsels.

Uit deze resultaten volgt dat beide nitrovasodilatoren een arteriolaire dilatatie kunnen veroorzaken, welke gepaard gaat met versterkte vasculaire pulsaties in de carotide circulatie van het varken. De uitgesproken arteriolaire dilatatie beperkt het effect van NO-donoren op arterioveneuze anastomosen, mogelijk als gevolg van een zogenaamd 'steal' fenomeen.

Bovendien lijkt de dilatatie van zowel extra- en intracraniële arteriën, en arterioveneuze anastomosen te leiden tot versterkte vasculaire pulsaties. De toegenomen pulsaties, meer nog dan de toename in de bloedstroom, zijn mogelijk mede verantwoordelijk voor de hoofdpijn veroorzaakt door nitrovasodilatoren.

Hoofdstuk 7 beschrijft een studie naar het effect van het neuropeptide, calcitonin gene-related peptide (CGRP) op de verdeling van de carotide bloedstroom en de hemodynamische variabelen in het genarcotiseerde varken. Dit neuropeptide kan een rol spelen in de pathofysiologie van migraine hoofdpijn en de vrijzetting van dit

peptide wordt, althans in sommige vaten, gemedieerd door NO. Humaan α-CGRP veroorzaakte een dosis-afhankelijke toename van de bloedstroom en conductantie in de arteria carotis, alsmede verhoogde vasculaire pulsaties. Deze effecten gingen gepaard met een daling van de systemische bloeddruk en een gelijktijdige toename van de hartfrequentie en het hartminuutvolume. De verhoogde carotide bloedstroom resulteerde in een toename van de capillaire bloedstroom naar voornamelijk extracerebrale weefsels waaronder de dura. De bloedstroom door arterioveneuze anastomosen in het carotide vaatbed bleef onveranderd.

De antimigraine middelen, dihydroergotamine en sumatriptan, reduceerden de verhoogde bloedstroom in de arteria carotis en het capillaire vaatbed zonder effect op de systemische conductie. De door α-CGRP geinduceerde toename in de weefsel doorbloeding werd in de meeste extracerebrale weefsels, met uitzondering van de dura, eveneens tegengegaan door deze middelen.

De resultaten uit deze experimenten tonen dat α-CGRP een arteriolaire dilatatie en een bloeddruk verlaging veroorzaakt in het varken. De daling van de systemische arteriële bloeddruk en de uitgesproken toename van de capillaire doorbloeding maskeert een eventueel effect van dit peptide op arterioveneuze anastomosen. De veranderingen van de bloedstroom in het carotis vaatbed van het varken, onder invloed van α-CGRP, kunnen worden tegengegaan met de antimigraine middelen dihydroergotamine en sumatriptan.

In *hoofstuk 8* worden de experimentele resultaten van dit proefschrift in het algemeen, en de mogelijke relatie tussen NO en de pathofysiologie van migraine in het bijzonder, besproken. Het merendeel van de hemodynamische effecten en de veranderingen in weefseldoorbloeding veroorzaakt door de NO-syntheseremmer L-NAME kunnen worden toegeschreven aan een afname van de basale NO-gemedieërde vasodilatoire tonus. Er bestaan geen aanwijzingen voor een antimuscarinerge werking van L-NAME in onze experimenten. De functionele tegenhangers van NO, welke verantwoordelijk zijn voor de toename van de systemische en lokale vaatweerstand, lijken in genarcotiseerde dieren voornamelijk te bestaan uit circulerende catecholamines.

De initiële stimulus welke verantwoordelijk is voor de verwijding van grote (extra)cerebrale vaten en arterioveneuze anastomosen tijdens de migraine aanval is grotendeels onbekend. Hoewel CGRP een potente vasodilator is, veroorzaakt dit neuropeptide geen hoofdpijn bij de mens en kon geen effect op de bloedstroom door

arterioveneuze anastomosen worden aangetoond in het varken. De experimenten met de NO syntheseremmer L-NAME en de verschillende NO-donoren suggereren daarentegen dat NO betrokken is bij de bloedstroomregulatie door deze vaatsegmenten en klinische experimenteel werk lijkt te duiden op een verhoogde gevoeligheid voor of activiteit van NO-gemedieërde processen in migraine patienten. Verschillende nitrovasodilatoren veroorzaken migraine-achtige hoofdpijn en een verwijding van extracerebrale arteriën en basale cerebrale arteriën. Deze effecten zijn meer uitgesproken in migraine patienten dan in gezonde proefpersonen. Hoofdpijn en lokale vaatverwijding is eveneens beschreven in migraine patienten voor de endotheel-afhankelijke vaatverwijder histamine. De verhoogde gevoeligheid van migraine patienten kan niet worden verklaard door veranderingen in de basale vrijzetting van NO door het vasculaire endotheel, omdat dit verschijnsel wordt waargenomen na zowel endotheel-afhankelijke als onafhankelijke vorming van NO. Een mogelijke bijdrage van nitroxiderge zenuwen aan de regulatie van de (extra)craniële bloedstroom is voorstelbaar.

In het algemeen kan worden gesteld dat de bijdrage van NO aan de regulatie van de lokale vaattonus en de regionale doorbloeding verschilt naar diersoort. Het vermogen van endogeen NO om de bloedstroom door carotide arterioveneuze anastomosen te beinvloeden lijkt relevant te zijn voor migraine hoofdpijn. Dit kan een aanzet vormen tot de ontwikkeling van een nieuwe therapie voor migraine.

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Marcel van Gelderen was born in Rottterdam, the Netherlands, on 14 october 1957. After attending the Adriaan Roland Holstcollege in Hilversum, where he passed his secundary school exam (atheneum B) in 1976, he studied Pharmacy at the University of Amsterdam till 1985. During his studies he worked for two years as a Research Assistant at the Department of Pharmacotherapy of the Faculty of Pharmacy (Head of Department: Professor Dr. P.A. van Zwieten). During a two year break (1986-1987), he worked as a cook at the Garden Hotel and Restaurant 'De Kersentuin' in Amsterdam. He finished his Pharmacy studies at the State University of Utrecht, where he obtained his M.Sc. in 1989. In januari 1990, he joined the Department of Pharmacology of the Erasmus University Rotterdam to start the research described in this thesis. After completing the experimental part of his thesis in 1994, he worked till september 1996 as a researcher at the Medical Insurance Board in Amstelveen. He is married to Saskia Boele and they have two children, Kimberley and Felix.

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List of abbreviations

A23187	calcium ionophore
AC	adenyl cyclase
ACE	angiotensin converting enzyme (kininase II)
Ach	acetylcholine
ADP	adenosine diphosphate
Bk	bradykinin
COX	cyclooxygenase
α-CGRP	calcitonin gene-related peptide
ECE	endothelin converting enzyme
ecNOS	endothelial NO-synthase, also denoted isoform III
EDCF	endothelium-derived contracting factor
EDHF	endothelium-derived hyperpolarizing factor
EDRF	endothelium-derived relaxing factor
ET	endothelin
ET_A	endothelin-receptor subtype A
ET_B	endothelin-receptor subtype B
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
cGMP	guanosine 3',5'-cyclic monophosphate
GC	guanylyl cyclase
5-HT	5-hydroxytryptamine, serotonine
iNOS	inducible NO-synthase, also denoted isoform II
L-NAME	N^G-nitro-L-arginine methyl ester
L-NNA	N^G-nitro-L-arginine
L-NMMA	N^G-monomethyl-L-arginine
L-OHARG	N^G-hydroxyl-L-arginine
LV dP/dt_{max}	left ventricular maximum pressure increase
μm	micrometer (10^{-6}m)
NADPH	nicotinamide-adenine dincleotide hydrophosphate
ncNOS	neuronal NO-synthase, also denoted isoform I
SOD	superoxide dismutase
TNFα	tumor necrosis factor-α
VIP	vasoactive intestinal polypeptide