Peripheral and cardiac consequences of diminished nitric oxide production

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1. Molnár G., Kaszaki J., Nagy S., Petri I., Balogh Á., Boros M.: Vazoaktív mediátorok szerepe a kolloid-krisztalloid volumen expanderek keringési hatásaiban. *Magyar Sebészet* 52: 215, 1999.
2. Kaszaki J, Eszlári E, Csonka E, Molnár G., Boros M: A kolloid-krisztalloid volumen expanzió következményei: hízósejtek és az endothelin-1 szerepe. *Magyar Sebészet* Suppl: 20, 2001.
3. Kaszaki J., Molnár G., Nagy S., Balogh Á., Boros M.: The role of endothelin and nitric oxide in the circulatory effects of colloid-crystalloid resuscitation fluids. J. Physiol. 526P: 127P, 2000.
4. Kaszaki J., Molnár G., Nagy S., Balogh Á., Boros M.: The role of vasoactive mediators in the circulatory effects of colloid-crystalloid volume expansion. European Surgical Research 32 (Supp1 1.): p57, 2000.
5. Molnár G., Kaszaki J., Eszlári E., Géczi T., Nagy S., Petri I., Boros M.: Interaction of endothelin and nitric oxide in cardiac effects of colloid-crystalloid volume expansion. Perfusion 8: 366, 2000.
6. Kaszaki J., Molnár G., Eszlári E., Csonka E., Nagy S., Petri I., Boros M.: The role of mast cells and endothelin-1 in the circulatory effects of colloid-crystalloid resuscitation fluids. European Surgical Research 33: 155, 2001.

CONTENTS

LIST OF ABBREVIATIONS

cAMP	-	cyclic adenosine monophosphate
cGMP	-	cyclic guanosine monophosphate
CI	-	cardiac index
cNOS	-	Ca^{2+}-dependent constitutive nitric oxide synthase
CO	-	cardiac output
CVP	-	central veous pressure
eNOS	-	endothelial nitric oxide synthase
ET	-	endothelin
ET-A	-	endothelin-A receptor
HR	-	heart rate
HS	-	hypertonic saline
HSD	-	hypertonic saline – dextran solution
iNOS	-	Ca^{2+}-independent inducible nitric oxide synthase
LV	-	left ventricular
LVD	-	left ventricular diameter
LVP	-	left ventricular pressure
MAP	-	mean arterial pressure
MPO	-	myeloperoxidase
NNA	-	N-ω-nitro-L-arginine
nNOS	-	neuronal nitric oxide synthase
NO	-	nitric oxide
NOS	-	nitric oxide synthase
TPR	-	total peripheral vascular resistance
XOR	-	xanthine oxidoreductase

SUMMARY

Nitric oxide (NO) is a broad-spectrum regulator of the cardiovascular system. A variable-level, but steady generation of NO is achieved by a family of isoenzymes. Although NO is regarded as predominantly a peripheral vasoactive mediator, the mammalian heart also expresses all three isoforms of NO synthase (NOS), and NO has several specific effects on the normal myocardium too. The role of endogenous NO in modulating myocardial contractility is still unclear, in part because of unknown, secondary effects of blocking NO release.

In the treatment of acute circulatory failure, small-volume resuscitation with hypertonic saline-dextran (HSD) solutions effectively improves the cardiovascular function and it has proved much more efficient than normotonic volume replacement. The primary target organ of fluid replacement is the vascular endothelium, a sensitive sensory and transmitter surface between the circulating blood and the wall of the vessels throughout the body, where several vasoactive mediators of endothelial origin are synthesized and released. Physiologically, the most important stimulus for the continuous formation of NO is the viscous drag (shear stress) generated by the streaming blood on the endothelial layer.

Given this background, the general aim of our studies was to examine and characterize the consequences of artificially diminished NO production in a large animal model by using the L-arginine analog nonselective NOS inhibitor N^{G}-nitro-L-arginine (NNA) on the central and peripheral circulatory patterns in normovolemia and during hyperosmotic colloid fluid therapies.

The results of Study I revealed that a diminished NO production leads to secondary reactions, whereby a mismatch between NO supply and demand in the cardiac muscle is translated into an increase in cardiac contractility. A reduced NO synthesis leads to increased superoxide production and preponderant vasoconstrictor effects, which decrease cardiac output (CO) and increase the myocardial contractility through an endothelin-A (ET-A) receptor-dependent mechanism. The results of Study II indicate that the generation of vasoactive mediators is involved in HSD-caused circulatory changes and in alterations in cardiac contractility through NO-dependent and independent mechanisms, and suggest the preponderance of ET-1 in this process. Furthermore it can be concluded that HSD is less effective in NO-deficient states.

These experimental data point to a suppressive, regulatory role for endogenous NO: it restrains or counteracts several mechanisms which would otherwise increase the cardiac contractility. The bioavailability of NO or its amount relative to other, vasoregulator, inotropic agents is of crucial importance.

1. INTRODUCTION

1.1. Generation of nitric oxide

NO is one of the few gaseous signaling molecules known and is additionally exceptional by virtue of the fact that it is a radical gas. It is a key vertebrate biological messenger, playing a role in a variety of biological processes. NO, known as the "endothelium-derived relaxing factor", is biosynthesized endogenously from L-arginine, oxygen and NADPH by various forms of NOS, leading to the formation of L-citrulline and NO (Palmer 1988). A variable-level, but steady generation of NO is achieved by a family of isoenzymes, including neuronal NOS (nNOS or NOS1), inducible NOS (iNOS/NOS2) and endothelial NOS (eNOS/NOS3). Constitutive types of NOS (cNOS) are Ca^{2+}/calmodulin-dependent, while iNOS is Ca^{2+}-independent. cNOS is responsible for the production of NO in a physiological context (Massion 2003). In contrast, iNOS produces NO under pathophysiological circumstances. nNOS was first described in the neurons of the central and peripheral nervous systems, while eNOS is present in the endothelium of blood vessels. It has been shown that all three types of NOS can be found in almost all cells of the body. NO is highly reactive (having a lifetime of a few seconds), yet diffuses freely across membranes. These attributes make NO ideal as a transient paracrine and autocrine signaling molecule (Moncada 1991).

1.2. Circulatory effects of nitric oxide

NO produced by NOS isoenzymes is a fundamental determinant of cardiovascular homeostasis: it regulates the systemic blood pressure, vascular remodeling and angiogenesis. NO contributes to vessel homeostasis by inhibiting vascular smooth muscle contraction and growth, platelet aggregation and leukocyte adhesion to the endothelium.

NO is produced by many cells in the body; however, its production by the vascular endothelium is particularly important in the regulation of the blood flow. As a short-lived powerful mediator, it has a rapid vasodilatatory effect. There are two basic pathways for the stimulation of cNOS, both of which involve the release of Ca^{2+} from subsarcolemmal storage sites. First, shearing forces acting on the vascular endothelium, generated by the blood flow, cause a release of Ca^{2+} and subsequent cNOS activation. Increases in blood flow therefore, stimulate NO formation (flow-dependent NO formation).

Second, endothelial receptors for a variety of ligands stimulate Ca^{2+} release and subsequent NO production (receptor-stimulated NO formation). The list includes receptors for acetylcholine, bradykinin, substance P, adenosine and many other vasoactive substances. These effects have been attributed to NO production by the eNOS; however, recent evidence

suggests that nNOS also has a distinct local role in the physiological regulation of the human microvascular tone (Seddon 2008).

NO is additionaly generated by phagocytes (monocytes, macrophages and neutrophils) as part of the human immune response. Phagocytes are armed with iNOS. In this way the immune system may regulate the armamentarium of phagocytes that play a role in inflammation and immune responses (Hibbs 1988). Under normal, resting conditions, the activity of iNOS is very low. The activity of iNOS is stimulated during inflammation by bacterial endotoxins (*e.g*. lipopolysaccharide) and cytokines such as tumor necrosis factor alfa and interleukins. During inflammation, the amount of NO produced by iNOS may be 1,000-fold greater than that produced by cNOS. This latter enzyme activity leading to high-output NO synthesis was initially perceived to act as a toxic defense mechanism associated with local tissue destruction in chronic inflammatory conditions (Steiner 1997). However, more recent investigations have linked a powerful protective activity toward cellular stress conditions with iNOS-derived NO synthesis (Buttery 1996, Suschek 1999).

1.3. Nitric oxide and cardiac function

Although NO is a primary determinant of the blood vessel tone, the recognition that all three isoforms of NOS are expressed in cardiomyocytes themselves has raised several intriguing questions regarding the signaling role of NO in the heart (Massion 2003).

The modulatory effects of NO on the cardiac function are undoubtedly complex (Kelly 1996, Shah 2000, Brutsaert 2003, Casadei 2003). They involve the enhancement of myocardial relaxation and the diastolic function (Shetty 1993, Paulus 1994, Casadei 2003), an increase or decrease in β-adrenergic responses (Balligand 1993, Keaney 1996), modulation of the force-frequency relationship (Cotton 2001) and the mediation of parasympathetic cholinergic effects (Han 1994). This is perhaps expected when one considers the versatility of NO biochemistry, the multiplicity of its intracellular targets (with sometimes opposite contractile influences), and the diversity of its cellular sources within the myocardium.

NO has beneficial actions on myocardial energetics and the left ventricular (LV) diastolic distensibility. In isolated cardiomyocytes, the administration of NO increases their diastolic cell length consistently with a shift to the right of the passive length-tension relation. This shift is explained by the cGMP-induced phosphorylation of troponin I, which prevents Ca^{2+}-independent diastolic cross-bridge cycling and concomitant diastolic stiffening of the myocardium (Shah 1994, Ito 1997). After adrenoreceptor stimulation of isolated cardiac muscle strips, the NO-induced relaxation effect was larger, probably because of the simultaneous phosphorylation of troponin I by cAMP-dependent and cGMP-dependent

protein kinases (Mohan 1996). A beneficial effect of high endomyocardial NO activity on the diastolic LV distensibility of the cardiomyopathic heart could also result from the prevention of endomyocardial fibrosis.

The effects of NO on the diastolic LV performance appear to be synergistic with its effects on myocardial energetics through prevention of myocardial energy wastage induced by LV contraction against late-systolic reflected arterial pressure waves (Rourke 1999) and through the prevention of diastolic LV stiffening, which is essential for the maintenance of adequate subendocardial coronary perfusion.

The exact role of endogenous NO production in the regulation of the cardiac contractility is still controversial (Balligand 1993, Brady 1993, Kojda 1996, Barouch 2002), mainly because of the disproportionate extrapolation from results relating to modulation of the NO output. The consequences of altered NO production may be studied with NO donors, NOS inhibition and genetically modified models, and there is *in vivo* evidence that the lack of NO leads to an increased myocardial contractility in dogs (Kaszaki 1996, Keaney 1996, Kilbourn 1997). In contrast, it has also been found that blockade of the NO/cGMP pathway with NOS inhibitors in rats and in isolated guinea pig cardiac myocytes points to the negative inotropic effect of NO (Kojda 1997, Barouch 2002). Similarly, studies with eNOS-deficient mouse strains have yielded contradictory results (Brunner 2001, Barouch 2002). While the positive inotropic response is enhanced in eNOS-deficient mice (Gyurko 2000, Barouch 2002), this effect was not present when isolated cardiomyocytes from eNOS knock-out mice were used (Godecke 2001). In the basal state or under low β-adrenergic stimulation, the effects of NO are bimodal, with a positive inotropic effect at low-level NO exposure, but a negative one at higher amounts (Casadei 2003, Massion 2003).

A possible explanation for these contrasting results could be that the cardiac reactions of NO are achieved via additional circulatory effectors. Under physiological conditions, peripherally released NO not only exerts effects on the vasculature, but also significantly influences the reactions of other cell types in the circulatory system. The roles and relationships of these secondary mechanisms in modulating the NO-linked myocardial responses are largely unknown, and it could be argued that NO may affect other pathways which lead indirectly to myocardial contractility changes.

At higher NO exposure in the case of sepsis, large concentrations of iNOS-derived NO overwrite the above mechanisms and steeply depress the cardiac contractile performance through cGMP-mediated and protein kinase G-mediated desensitization of cardiac

myofilaments (Tavernier 2001). A similar mechanism may depress the basal cardiac function in mice overexpressing large amounts of eNOS (Brunner 2001).

As a radical gas, NO has a lifetime of only a few seconds. Its bioavailability on its specific targets depends greatly on certain bioactive agents. Free radical scavengers such as myoglobin, particularly abundant in the cardiomyocytes, inhibit NO availability. Accordingly, the effects of NO donors and endogenous NO on the contractile force are enhanced in hearts from myoglobin-deficient mice (Flogel 2001). Reactive oxygen intermediates and NO mutually influence the bioavailability of each other, production and actions in a multi-level manner. They create peroxynitrite, a known inhibitor of NOS activity (Sheehy 1998, Ovadia 2002). This compound has also been associated with a depressed cardiac pump function through myocardial protein nitration.

1.4. Pathophysiological consequences of diminished nitric oxide production

The integrity of the endothelium is essential for this flow-mediated vasodilation (Davies 1993), which is largely due to the release of NO, although in some vascular beds prostacyclin, endothelium-derived hyperpolarizing factor, or direct activation of an K^+ channels may contribute (Pohl 1986). Furthermore, it has been demonstrated that endothelial dysfunction is associated with reduced NO bioavailability in the cardiovascular system (Dixon 2005).

Studies have identified hemodynamic shear stress as an important determinant of endothelial function and phenotype. Arterial-level shear stress (>15 dyne cm^{-2}) induces endothelial quiescence and an atheroprotective gene expression profile, while low shear stress (<4 dyne cm^{-2}), which is prevalent at atherosclerosis-prone sites, stimulates an atherogenic phenotype (Malek 1999).

Disturbances of flow-mediated vasodilation may have pathophysiological consequences. Humans with atherosclerosis, diabetes or hypertension often exhibit impaired NO pathways. In patients with coronary artery disease and impairment of the endothelial vasodilatory function, increases in blood flow through the diseased coronary artery cause a paradoxical vasoconstriction, which can contribute to myocardial ischemia during exercise or mental stress (Pedersen 1997). Proliferation of vascular smooth muscle is seen in states of endothelial dysfunction that are characterized by a reduced release of NO, providing further support for its role in regulating the vascular structure. It is often observed following balloon injury that the myointimal proliferation continues even after the vessel wall has been resurfaced by endothelium. These cells appear to release less NO, as manifested by an attenuation of endothelium-dependent relaxations in these vessels (Weidinger 1990). In

hypercholesterolemic animals and man, endothelium-dependent relaxation is reduced and vasoconstriction is enhanced. This is largely due to a reduction in NO activity and not to the physical absence of the endothelium (Drexler 1991).

Increasing evidence indicates that endothelium-derived NO may act to oppose the hypercholesterolemia-induced alterations in the endothelial redox state. In the normocholesterolemic rabbit thoracic aorta, there is a basal production of superoxide anion by non-endothelial cells (Ohara 1993). Exogenous NO donors significantly reduce the generation of superoxide anion. When neutrophils are exposed to NO, their ability to generate superoxide anion is limited (Clancy 1992). Specifically, in the context of atherogenesis, NO has been demonstrated to inhibit thrombosis, cytokine-induced vascular cell adhesion molecule-1 expression, leukocyte adhesion to the endothelium, and smooth muscle proliferation and migration (Malek 1995). Together, these observations suggest that NO is an important atheroprotective molecule (Topper 1996).

1.5. Significance of hypertonic fluid resuscitation in circulatory disturbances

Adequate volume replacement is of great significance in the therapy of the circulatory disturbances in cases of both perioperative and intensive care attendance. The main goal of fluid therapy is to ensure an adequate oxygen supply for the organs. The individual volume therapy during surgical procedures in normovolemic cases often plays a crucial role in successful management. This essential role is also important throughout the whole perioperative phase (Vollmar 2004).

Small-volume resuscitation using hyperosmotic, colloid-crystalloid solutions effectively improves the cardiovascular function and it has proved much more efficient than normotonic volume replacement (Velasco 1980, Kramer 1986, Armistead 1989). Hypertonic saline (HS) solutions were originally developed for prehospital use and designed to replace larger volumes of isotonic solutions. The reasons for using a standard dose of 4 ml kg^{-1} HS or hypertonic saline-dextrane (HSD), seem to be based more on practicality rather than on any true physiologic concept.

Hyperosmotic solutions cause a plasma volume expansion by withdrawing water from the extravascular space, reducing the extravascular pressure, increasing the circulating volume and creating favorable histological pressure conditions at the level of the capillaries (Frohlich 1966). As a result, they effectively improve the cardiovascular function and reduce the total peripheral resistance, thereby enhancing the capillary circulation, and improving the perfusion of hypoperfused organs. In the long run, hyperosmotic solutions increase the amount of

oxygen available in the body through increasing the blood pressure and CO and reducing the resistance.

Dextran-based hyperosmotic solutions are known to be effective in restoring the macrohemodynamics (Kramer 1986, Dries 1996). By adding them to HS solution, this effect can be enhanced and, at the same time, swelling of the endothelial cells can be prevented (Mazzoni 1990). The most beneficial hemorheological properties of dextran are the inhibition of erythrocyte and thrombocyte aggregation and adhesion, changing the surface polarization of erythrocytes and endothelial cells (Rutherford 1988), and decreasing the activity of Factor VIII (Bergentz 1978). According to recent studies, high molecular weight dextran (dextran-500) attaches to the endothelial surface more effectively than do low molecular weight dextrans (dextran-20, -40 or -70), which are commonly used in medicine (Gonzalez-Castillo 2002). The protective role of low molecular weight dextran may therefore become questionable.

Since the primary target organ of fluid replacement is the vascular endothelium, it can play a very significant role in resuscitation. This sensitive sensory and transmitter surface between the circulating blood and the wall of the vessels (Hecker 1993) througout the body is the site where several vasoactive mediators of endothelial origin are synthesized and released (Miller 1992, Kuchan 1993). Infused HSD solution presents a considerable mechanical stimulus for the endothelial cells, partly due to increase in the shear stress affecting the surface, and also because of the hyperosmotic effect. Several experimental and clinical studies have investigated the efficacy, dosages and infusion rates of different hyperosmotic solutions: primarily 7.5% HS administered alone or in combination with dextran or hetastarch (Wade 1991, Kreimeier 1993). Although the clinical data do not indicate that the administration of hypertonic solutions increases mortality in the clinical setting (Wade 1997, Younes 1997), the results of experimental studies suggest that the administration of HS exacerbates bleeding from injured vessels and leads to early death in anesthetized animals (Vassar 1990, Krausz 1995).

2. MAIN GOALS

The general aim of our studies was to analyze the effects of decreased NO production on the central and peripheral circulatory patterns in normovolemia and during fluid therapies. Since the nonspecific inhibition of NOS is linked to several secondary reactions, our **primary goal** was to devise a study design that helps to identify a causal chain mechanism relevant to a single cause - multiple effect phenomenon. This approach was used to establish NO-linked contractility responses.

In a second series of experiments, a **further goal** was to examine the clinical consequences of a rapid volume expansion after the administration of a hypertonic–hyperoncotic solution. To this end, we employed a 7.2% hypertonic saline–10% dextran-40 (HSD) solution to induce rapid changes in the cardiac hemodynamics with or without non-specific NOS inhibitor pretreatment.

- Study I characterized the myocardial contractility consequences of pharmacologically decreased NO production in a large animal model by using the L-arginine analog aspecific NOS inhibitor N^G-nitro-l-arginine (NNA) and the NO dependence of several secondary variables was examined.
- Study II was designed to explore a connection between the increased peripheral flow-induced changes and cardiac contractility alterations. In this, setup we outlined the role of NO in the HSD-evoked hemodynamic changes, and investigated the effects of small-volume resuscitation in conditions associated with diminished NO production.

3. MATERIALS AND METHODS

The experiments were performed in adherence to the NIH guidelines for the use of experimental animals. The study was approved by the Ethical Committee for the Protection of Animals in Scientific Research at the University of Szeged.

3.1. Surgical preparation

The experiments were performed on a total of 52 inbred mongrel dogs (average weight 17 ± 2.8 kg). Anesthesia was induced with sodium pentobarbital (30 mg kg^{-1} iv) and sustained with 0.2 mg kg^{-1} h^{-1} supplementary doses. After intubation of the trachea, the animals were mechanically ventilated with room air (Harvard Apparatus, South Natick, MA, U.S.A.). The left femoral artery and vein were cannulated for the measurement of mean arterial pressure (MAP) and the administration of drugs and fluids, respectively. Blood gas parameters were regularly controlled throughout the experiments. The animals received 15 ml kg^{-1} h^{-1} Ringer's lactate infusion during Study I. In Study II, an identical dose of Ringer's lactate was given until the start of HSD treatment. A Swan-Ganz thermodilution catheter (Corodyn TD-E-N, 5011-110-7Fr; Braun Melsungen AG, Melsungen, Germany) was positioned into the pulmonary artery via the right femoral vein to measure and calculate the CO and the cardiac index (CI).

In Studies I and II, an inflatable balloon-catheter (Foley catheter, 14Fr, Kendall Company Ltd., Basingstoke, U.K.) was introduced into the inferior caval vein via the left jugular vein. The filling volume was 10 ml. A catheter tip micromanometer (Millar Instruments Inc., Houston, TX, U.S.A.) was introduced into the left ventricle through the left internal carotid artery to monitor the LV pressure (LVP). A left thoracotomy was performed at the sixth intercostal space and the pericardium was opened. A pair of ultrasonic dimension crystals (3 MHz, ID-4, Custom Transducers, Poway, CA, U.S.A.) were sutured onto the anterior and posterior walls of the left ventricle, opposite each other, using an atraumatic surgical technique for measurement of the LV diameter (LVD). The thoracic cavity was revised and the chest wall was closed in four layers. The air was removed from the thorax; the animals were then breathing spontaneously. Their body temperature was maintained at 38 ^{o}C with a homeothermic blanket. At the end of the experiments, a myocardial tissue biopsy sample was taken from the left ventricle and the animals were killed with an overdose of pentobarbital.

In Studies II, to measure coronary blood flow, an ultrasonic flow probe (Transonic Systems, Ithaca, NY, USA) was placed around the left circumflex coronary artery supplying the left ventricle.

3.2. Macrohemodynamic measurements

All hemodynamic signals (pressures, LVP, LVD and coronary flow) were registered with a computerized data-acquisition system (SPEL Advanced Haemosys 2.72, Experimetria Ltd., Budapest, Hungary). The MAP and central venous pressure (CVP) were monitored with Statham P23Db transducers. The heart rate (HR) was calculated from the MAP curve. The CO was determined by thermodilution, using a Cardiostar CO-100 computer (Experimetria Ltd., Budapest, Hungary), normalized for body weight and expressed as CI (ml kg^{-1} min^{-1}). The total peripheral vascular resistance (TPR) was calculated via the standard formula.

The ultrasonic dimension crystals were connected to a sonomicrometer (Triton Technology, Inc., San Diego, CA, U.S.A.). Via the LVP and LVD signals, the end-systolic elastance, as a parameter of the LV myocardial contractility, was estimated from the slope of the end-systolic pressure *vs* diameter relationship (Goldfarb 1982, Kaszaki 1996) with a computer program developed by our group. The inferior caval vein was briefly occluded by a balloon catheter, and the pressure *vs* diameter loops were registered for 8 s. The end-systolic points of the loops (which can be fitted to a sigmoid curve) were recorded. The linear part of the curve was selected on the basis of the lowest variance, and a straight line was fitted to the selected points. The computer program calculated contractility as the slope of the end-systolic pressure *vs* diameter relationship, and the variance of fitting was determined. The calculation was based on a minimum of 8 cardiac cycles.

3.3. Biochemical measurements

Plasma ET-1 measurements

Two-ml blood samples were drawn from the jugular vein into chilled polypropylene tubes containing EDTA (1 mg ml^{-1}) and aprotinin (Trasylol, Bayer, Leverkusen, Germany) (500 KIU/mL) before and after ETR-P1/fl peptide and NNA infusions, and at the end of the observation period. The blood samples were centrifuged at 1200g for 10 min at 4 ^{o}C. The plasma samples were then collected and stored at -70 ^{o}C until assay. Plasma samples were analyzed for ET-1 with an ELISA kit (Biomedica, Vienna, Austria). According to the manufacturer, the cross-reactivity with ET-1 and ET-2 was 100%.

Plasma nitrite/nitrate level measurements

The levels of plasma nitrite/nitrate (NO_x), stable end-products of NO, were measured by the Griess reaction. The assay depends on the enzymatic reduction of nitrate to nitrite, which was then converted into a colored azo compound detected spectrophotometrically at 540 nm (Moshage 1995).

NOS activity measurements

NO formation in cardiac tissues was measured via the conversion of [^{3}H]L-citrulline from [^{3}H]L-arginine according to the method of Szabó (1993). Briefly, heart biopsies kept on ice were homogenized in phosphate buffer (pH 7.4) containing 50 mM Tris-HCl, 0.1 mM EDTA, 0.5 mM dithiotreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg ml^{-1} soybean trypsin inhibitor and 10 μg ml^{-1} leupeptin. The homogenate was centrifuged at 4 ^{o}C for 20 min at 24,000g and the supernatant was loaded into centrifugal concentrator tubes (Amicon Centricon-100; 100,000 MW cut-off ultrafilter). The tubes were centrifuged at 1000g for 150 min and the concentrated supernatant was washed out from the ultrafilter with 250 μl homogenizing buffer. The samples were incubated with a cation-exchange resin (Dowex AG 50W-X8, Na^+ form) for 5 min to deplete endogenous L-arginine. The resin was separated by centrifugation (1500g for 10 min) and the supernatant containing the enzyme was assayed for NOS activity.

For the Ca^{2+}-dependent NOS (cNOS) activity, 50 μl enzyme extract and 100 μl reaction mixture (pH 7.4, containing 50 mM Tris-HCl buffer, 1 mM NADPH, 10 μM tetrahydrobiopterine, 1.5 mM $CaCl_2$, 100 U ml^{-1} calmodulin and 0.5 μCi [^{3}H]L-arginine (Amersham U.K., specific activity 63 Ci mmol^{-1})) were incubated together for 60 min at 37 ^{o}C. The reaction was stopped by the addition of 1 ml ice-cold HEPES buffer (pH 5.5) containing 2 mM EGTA and 2 mM EDTA. Measurements were performed with the NOS inhibitor NNA (3.2 mM) to determine the extent of [^{3}H]L-citrulline formation independent of the NOS activity. Ca^{2+}-independent NOS activity (iNOS) was measured without Ca-calmodulin and with EGTA (8 mM). 1 ml reaction mixture was applied to Dowex cation-exchange resin (AG 50W-X8, Na^+ form) and eluted with 2 ml distilled water. The eluted [^{3}H]L-citrulline activity was measured with a scintillation counter (Tri-Carb Liquid Scintillation Analyzer 2100TR/2300TR, Packard Instrument Co, Meriden, CT, U.S.A.). Protein contents of samples were determined by the Lowry method.

Whole blood superoxide production

Superoxide production was assessed by the lucigenin-enhanced chemiluminescence assay of Zimmermann *et al.* (Zimmermann 1991). Briefly, 10 μl blood samples were added to 1 ml Hank's balanced solution (PAA Cell Culture Company) at 37 ^{o}C. After addition of 100 μl lucigenin (15 μM), the chemiluminescence response was measured (as relative light units) over a period of 30 min, using a Lumat LB9507 luminometer (Berthold, Germany).

Determination of myocardial xanthine oxidoreductase (XOR) activity

Heart biopsies kept on ice were homogenized in phosphate buffer (pH 7.4) containing 50 mM Tris-HCl, 0.1 mM EDTA, 0.5 mM dithiotreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg ml^{-1} soybean trypsin inhibitor and 10 μg ml^{-1} leupeptin. The homogenate was centrifuged at 4 ^{o}C for 20 min at 24,000g and the supernatant was loaded into centrifugal concentrator tubes. The activity of XOR was determined in the ultrafiltered supernatant by fluorometric kinetic assay based on the conversion of pterine to isoxanthopterine in the presence (total XOR) or absence (xanthine oxidase activity) of the electron acceptor methylene blue (Beckman 1989).

Myocardial myeloperoxidase (MPO) activity measurement

The MPO activity, as a marker of tissue polymorphonuclear leukocyte infiltration, was measured via cardiac muscle biopsies (Kuebler 1996). Briefly, the sample was homogenized with Tris-HCl buffer (0.1 M, pH 7.4) containing 0.1 mM phenylmethylsulfonyl fluoride to block tissue proteases, and then centrifuged at 4 ^{o}C for 20 min at 2000g. The MPO activities of the samples were measured at 450 nm (UV-1601 spectrophotometer, Shimadzu, Japan) and the data were referred to the protein content.

3.4. Experimental protocols

In Study I, the animals were randomly allocated to one or other of three groups. Surgery was followed by a recovery period for cardiovascular stabilization. Baseline variables were determined during a 15-min control period. Group 1 (n=7) was treated with 0.9% saline iv, while in Groups 2 and 3 (n=7 each) the animals received 4 mg kg^{-1} NNA (Sigma Chem. U.S.A.) in 2 ml kg^{-1} saline during a 15-min iv infusion. The animals in Group 3 were additionally pretreated (100 nmol kg^{-1} iv bolus in 1.5 ml kg^{-1} saline) with the selective ET-A receptor antagonist ETR-P1/fl peptide (VLNLCALSVDRYRAVASWRVI; Kurabo Ltd., Osaka, Japan) (Baranyi 1995, Baranyi 1998) 30 min before NNA treatment. The animals were observed for 135 min after the end of the treatment period; hemodynamic measurements were performed every 30 min.

In Study II, surgery was followed by a recovery period for cardiovascular stabilization, and baseline variables were then determined during a 30-min control period. The animals were randomly allocated to one or other of three groups. Group 4 (n = 10), which as control, was treated with 0.9% saline (4 ml kg^{-1}), while Groups 5 (n = 7) and 6 (n = 7) were infused iv with 4 ml kg^{-1} HSD during 15 min. The solution was prepared from isotonic 10% dextran-40 (Baxter, Munich, Germany) and 7.2% NaCl solution. The animals in Group 6 were additionally treated with 4 mg kg^{-1} NNA (Sigma Chem. U.S.A.) in 2 ml kg^{-1} saline during a 5-

min iv infusion 15 min before HSD treatment. The beginning of HSD infusion served as the zero point of the experiments, and the animals were observed for a further 120 min in all groups.

3.5. Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Nonparametric methods were used. Friedman repeated measures analysis of variance on ranks was applied within the groups. Time-dependent differences from the baseline (time 0) for each group were assessed by Dunn's method, and differences between groups were analyzed with Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn's method for pairwise multiple comparison. In the Figures and Table 1, median values and 75th and 25th percentiles are given. P values < 0.05 were considered significant.

4. RESULTS

4.1. Study I: Cardiac and peripheral effects of NNA treatment

Initial effects of surgery

In the Group 1, there were no significant hemodynamic changes as compared with the baseline values, and the plasma NO_x and ET-1 levels did not change significantly during the 180-min observation period.

Hemodynamic effects of NNA infusion

The infusion of 4 mg/kg NNA resulted in sustained increases in MAP (Figure 1) and CVP (data not shown). Nonspecific NOS inhibition caused an approximately 25% decrease in CI (Figure 2), parallel with a gradual decrease in heart rate (HR) (Figure 3). The total peripheral vascular resistance (TPR) was increased nearly 2-fold as compared with the baseline values 105 min after NNA treatment (Figure 4).

Nonspecific NOS inhibition significantly reduced the difference between the LV diastolic and systolic diameters (as a percentage of the baseline) (Figure 5) and caused a significant increase in myocardial contractility up to the end of the observation period (Figure 6).

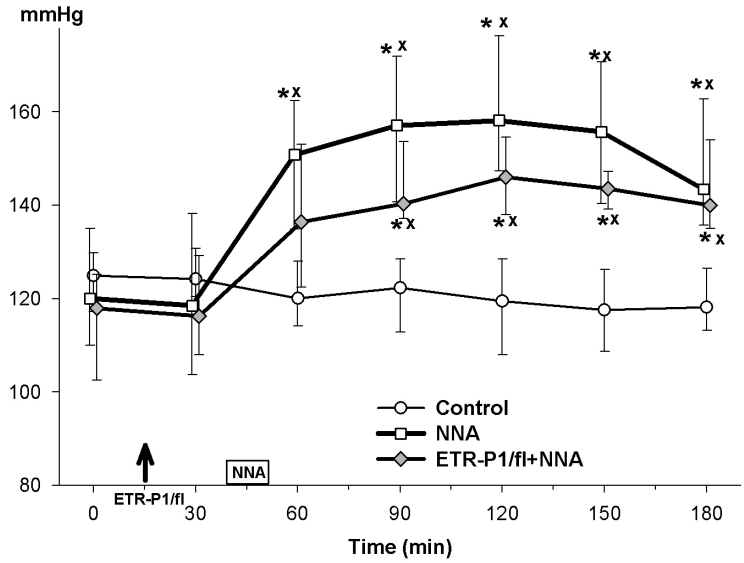

Figure 1. Changes in MAP in the control group (Group 1; circles), the NNA-treated group (squares) and the ETR-P1/fl+NNA-treated group (diamonds). Data are expressed as medians ± 25th and 75th percentiles. * $P < 0.05$ within group; ^{X} $P < 0.05$ between groups *vs* saline-treated control group values, $^{\#}$ $P < 0.05$ between groups *vs* NNA-treated group values.

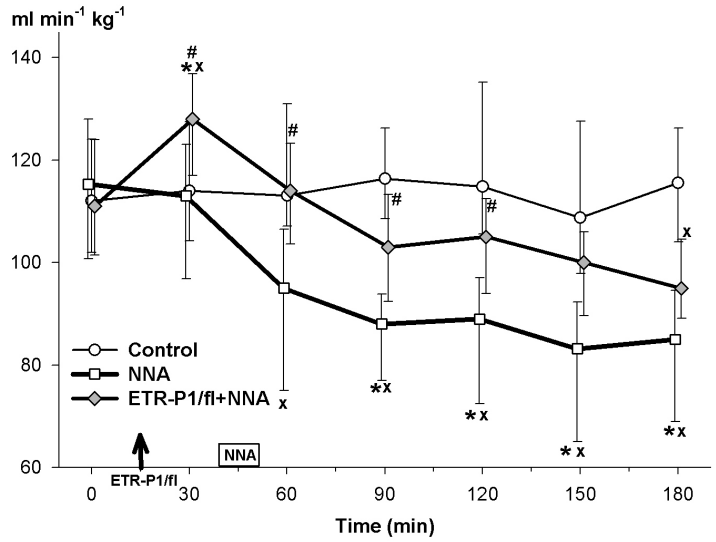

Figure 2. Changes in CI in the control group (Group 1; circles), the NNA-treated group (squares) and the ETR-P1/fl+NNA-treated group (diamonds). Data are expressed as medians ± 25th and 75th percentiles. * $P < 0.05$ within group; ^{X} $P < 0.05$ between groups *vs* saline-treated control group values, $^{\#}$ $P < 0.05$ between groups *vs* NNA-treated group values.

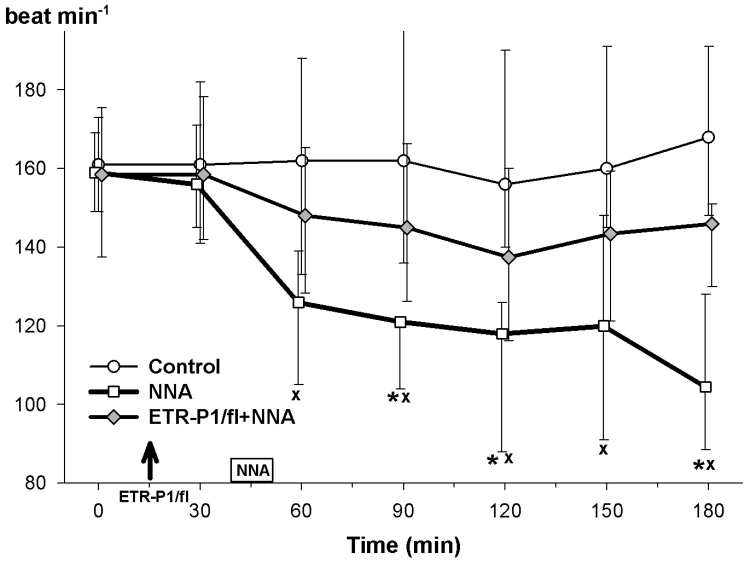

Figure 3. Changes in HR in the control group (Group 1; circles), the NNA-treated group (squares) and the ETR-P1/fl+NNA-treated group (diamonds). Data are expressed as medians ± 25th and 75th percentiles. * $P < 0.05$ within group; ^{X} $P < 0.05$ between groups *vs* saline-treated control group values, $^{\#}$ $P < 0.05$ between groups *vs* NNA-treated group values.

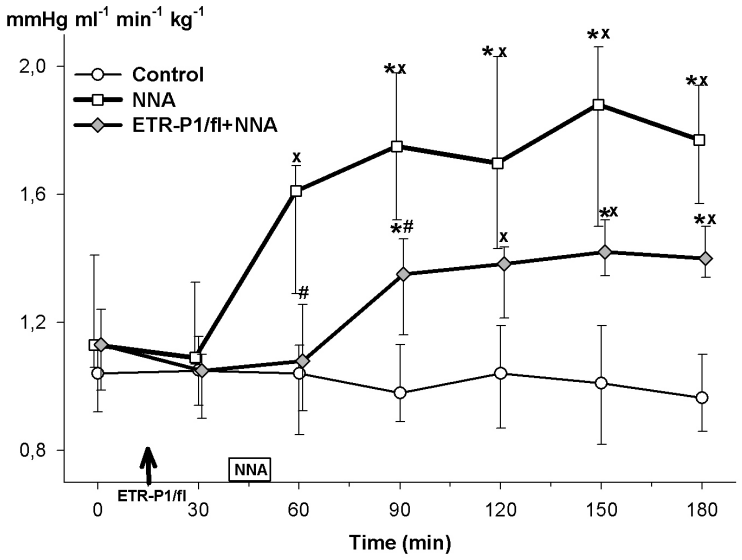

Figure 4. Changes in TPR in the control group (Group 1; circles), the NNA-treated group (squares) and the ETR-P1/fl+NNA-treated group (diamonds). Data are expressed as medians ± 25th and 75th percentiles. * $P < 0.05$ within group; ^{X} $P < 0.05$ between groups *vs* saline-treated control group values, $^{\#}$ $P < 0.05$ between groups *vs* NNA-treated group values.

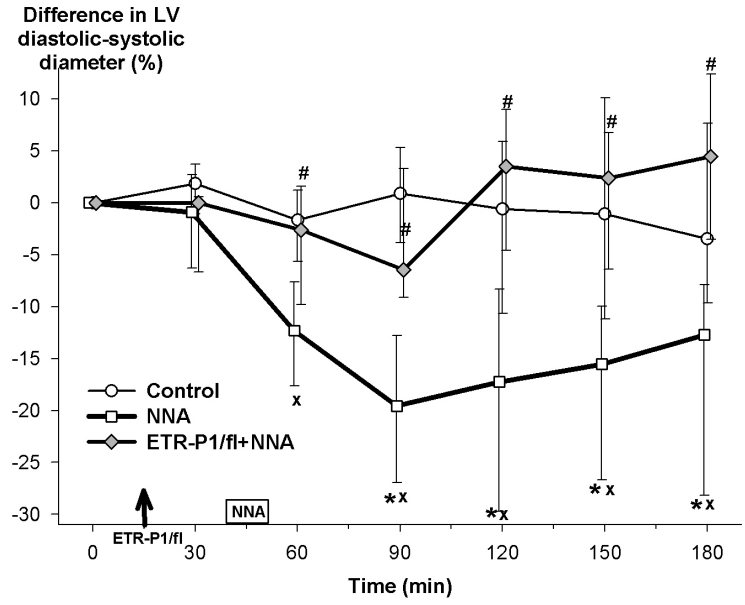

Figure 5. Changes in LV diastolic-systolic diameter difference in the control group (Group 1; circles), the NNA-treated group (squares) and the ETR-P1/fl + NNA-treated group (diamonds). * $P < 0.05$ within group; $^{X}P < 0.05$ between groups *vs* saline-treated control group values; $^{\#}$ $P < 0.05$ between groups *vs* NNA-treated group values.

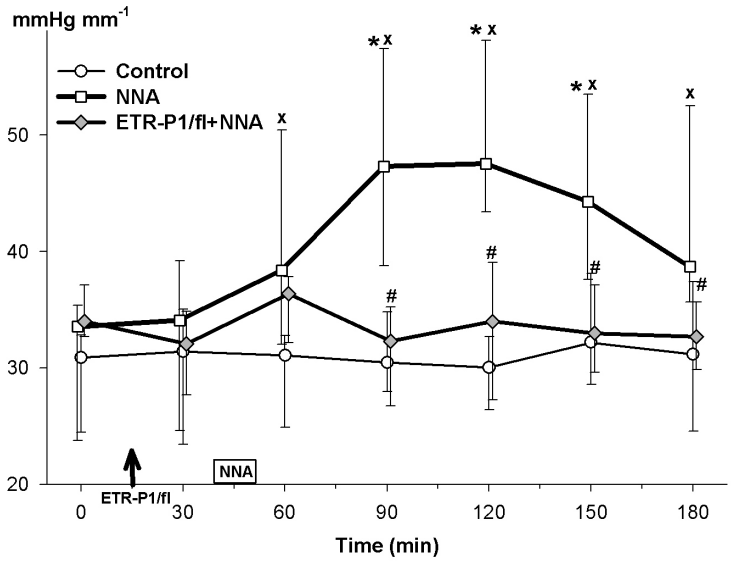

Figure 6. Changes in myocardial contractility in the control group (Group 1; circles), the NNA-treated group (squares) and the ETR-P1/fl + NNA-treated group (diamonds). * $P < 0.05$ within group; ^{X} $P < 0.05$ between groups *vs* saline-treated control group values; $^{\#}$ $P < 0.05$ between groups *vs* NNA-treated group values.

Hemodynamic effects of endothelin-A receptor antagonist pretreatment

The ETR-P1/fl peptide pretreatment mitigated the NNA-induced MAP and CVP elevations, but the differences between the values for the NNA and ETR-P1/fl peptide+NNA groups were statistically not significant (Figure 1). The cardiac effects of ET-A receptor antagonist pretreatment included an immediate, significant increase in CI at 30 min of the experiment, and the NNA-induced CI decrease was then significantly reduced (Figure 2). The pretreatment mitigated the NNA-induced decrease in HR, but the difference between the NNA and ETR-P1/fl peptide+NNA groups was statistically not significant (Figure 3). The pretreatment effectively antagonized the NOS inhibition-induced TPR elevation in the first 60 min after NNA administration (Figure 4).

However, ETR-P1/fl peptide pretreatment significantly inhibited the NNA-induced decrease in the LV diastolic-systolic diameter difference (Figure 5) and the NNA-induced elevation in myocardial contractility (Figure 6).

Biochemical changes in blood and myocardial tissue

The plasma ET-1 concentration gradually rose to approximately 1.5-fold following NNA infusion and remained significantly higher than in the control group (Group 1) up to 120 min in the observation period (Figure 7). The NOS inhibitor treatment caused a

significant decrease in plasma NO_x level at 90 min (Figure 8). Concomitantly, an approx. 5-fold increase in blood superoxide production was observed (Figure 9).

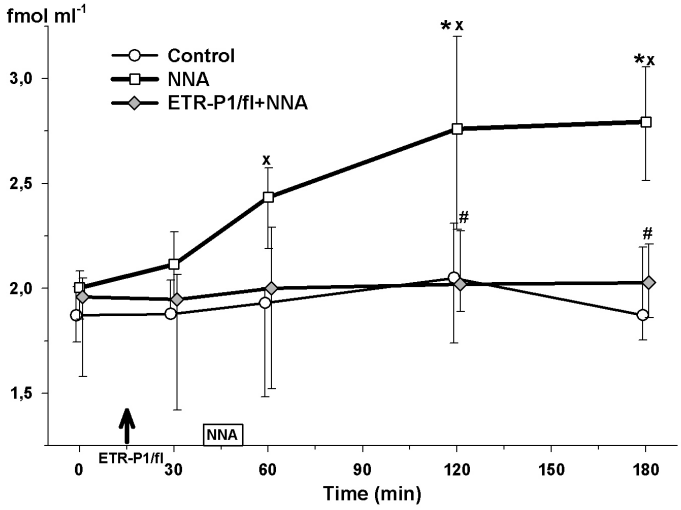

Figure 7. Changes in plasma ET-1 level in the control group (Group 1; circles), the NNA-treated group (squares) and the ETR-P1/fl+NNA-treated group (diamonds). * $P < 0.05$ within group; ^{X} $P < 0.05$ between groups *vs* saline-treated control group values; $^{\#}$ $P < 0.05$ between groups *vs* NNA-treated group values.

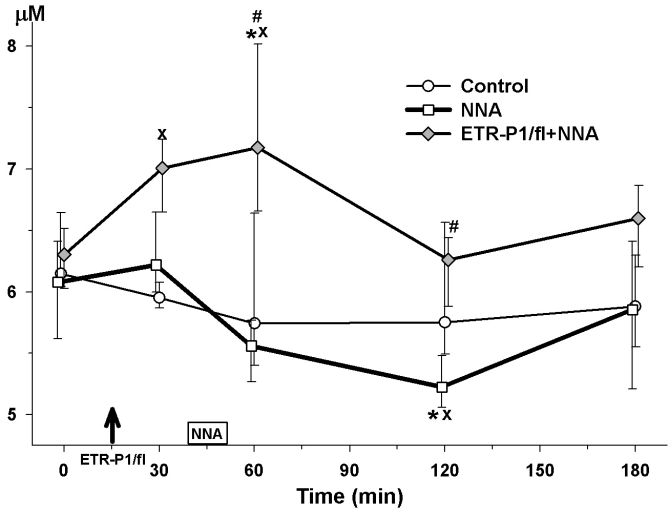

Figure 8. Changes in plasma NO_x level in the control group (Group 1; circles), the NNA-treated group (squares) and the ETR-P1/fl+NNA-treated group (diamonds). * $P < 0.05$ within group; ^{X} $P < 0.05$ between groups *vs* saline-treated control group values; $^{\#}$ $P < 0.05$ between groups *vs* NNA-treated group values.

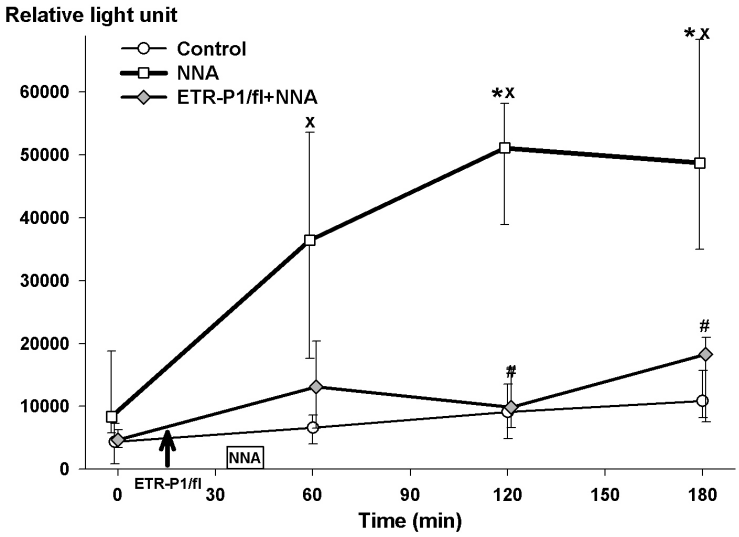

Figure 9. Changes in superoxide production of whole blood in the control group (Group 1; circles), the NNA-treated group (squares) and the ETR-P1/fl+NNA-treated group (diamonds). * $P < 0.05$ within group; ^{X} $P < 0.05$ between groups *vs* saline-treated control group values; $^{\#}$ $P < 0.05$ between groups *vs* NNA-treated group values.

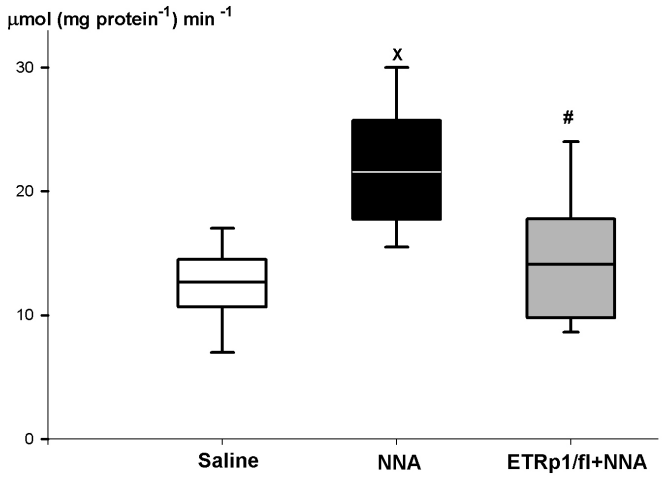

Figure 10. XOR activities in myocardial tissue 135 min after treatment in the saline-treated control group (empty box), the NNA-treated group (black box) and the ETR-P1/fl peptide+NNA-treated group (gray box) animals. The plots demonstrate the median values and the 25th (lower whisker) and 75th (upper whisker) percentiles. ^{X} $P < 0.05$ between groups *vs* saline-treated control group values. $^{\#}$ $P < 0.05$ between NNA-treated and ETR-P1/fl peptide+NNA-treated group values.

The ETR-P1/fl peptide pretreatment prevented the NNA-induced increase in plasma ET-1 level throughout the observation period (Figure 7) and significantly inhibited the NNA-induced superoxide production in the blood (Figure 9). The plasma NO_x level was significantly higher following the ETR-P1/fl peptide pretreatment until the end of the observation period as compared with the NNA-only group (Figure 8).

The myocardial XOR activity was significantly elevated 135 min after NNA treatment as compared with the control group (Figure 10). The ET-A antagonist pretreatment prevented this late consequence of the NNA infusion.

4.2. Study II: Early and late effects of nitric oxide on small-volume resuscitation

The concentration of the HSD solution and the optimal conditions for the volume expander protocol were determined in pilot studies (data not shown). In the control group (Group 4), there were no significant hemodynamic changes as compared with the baseline values, and the mediator level did not change significantly during the120-min observation period.

Hemodynamic effects of HSD infusion with or without NNA treatment

The HSD-induced peripheral circulatory reaction was characterized by transient MAP and CVP increases (Figure 11 and Table 1) and a biphasic change in TPR: an initial decrease was followed by a return to the baseline during the early phase of the postinfusion period, and the TPR was significantly elevated at 120 min in the postinfusion period (Figure 12). NNA pretreatment significantly elevated the MAP before HSD infusion, but there was no significant difference between the two HSD-infused groups (Figure 11). This pretreatment caused significant, long-lasting elevations in CVP and TPR relative to the HSD and control groups (Table 1 and Figure 12).

Table 1. Effects of saline, HSD or NNA+HSD pretreatment on CVP (mmHg); * $P < 0.05$ within group; ^{X} $P < 0.05$ between groups vs saline-treated control group values; $^{\#}$ $P < 0.05$ between groups vs HSD-treated group values.

	-30 min	-1 min	15 min	30 min	60 min	120 min
Control Median 25p, 75p	1.5 1.1; 2.0	1.7 1.4; 2.1	1.9 1.6; 2.4	1.9 1.4; 2.6*	2.0 1.7; 2.6	1.8 1.5; 2.5
HSD Median 25p, 75p	1.7 1.3; 2.1	1.9 1.7; 2.1	3.8*x 3.6; 4.1	2.9* 2.4; 3.6	2.4 2.1; 3.1	2.2 2.0; 2.5
NNA+HSD Median 25p, 75p	1.4 1.2; 1.9	3.3 1.7; 3.5	5.9*x# 4.1; 5.4	4.7*x# 4.1; 5.4	4.1*x# 3.4; 5.1	4.0*x# 3.1; 4.4

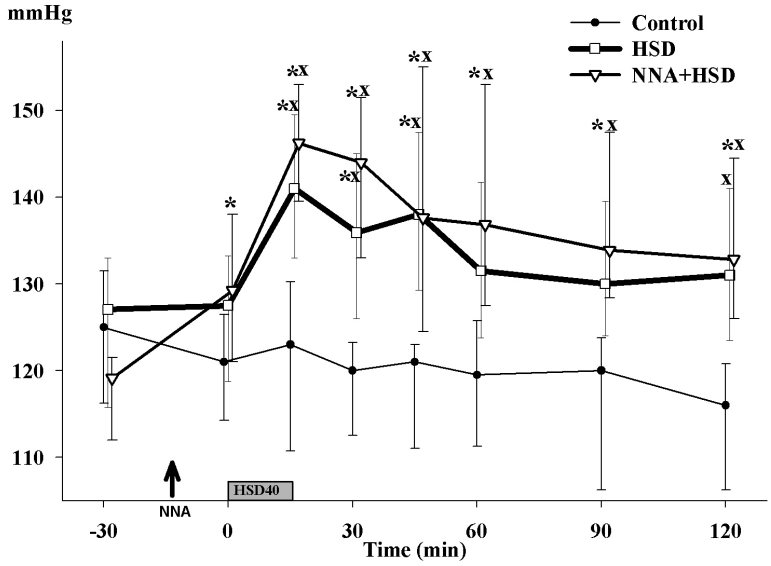

Figure 11. Changes in MAP in the saline-treated control group (Group 4; circles), the HSD-treated group (squares) and the NNA+HSD treated group (triangles). * $P < 0.05$ within group; ^{X} $P < 0.05$ between groups *vs* saline-treated control group values.

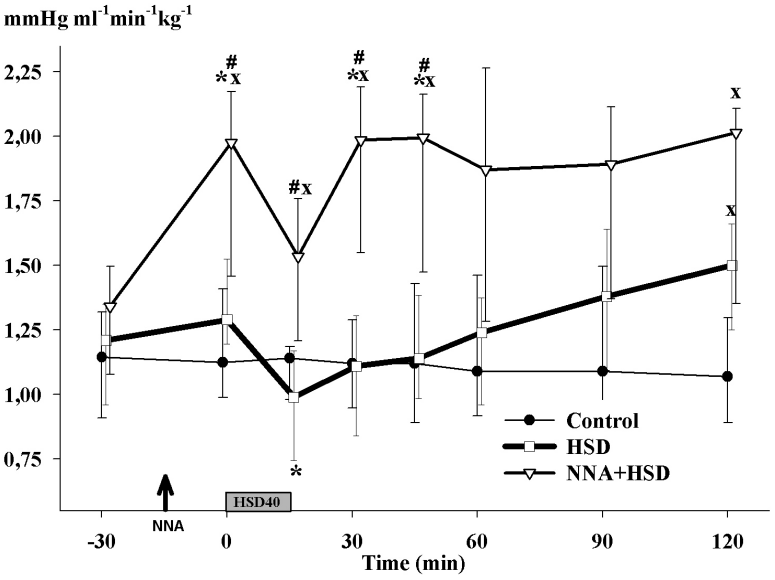

Figure 12. Changes in TPR in the saline-treated control group (Group 4; circles), the HSD-treated group (squares) and the NNA+HSD treated group (triangles). * $P < 0.05$ within group; ^{X} $P < 0.05$ between groups *vs* saline-treated control group values; $^{\#}$ $P < 0.05$ between groups *vs* HSD-treated group values.

The cardiac consequences of the HSD-induced volume loading included a significant increase in CI (Figure 13), together with a gradually elevated HR (Figure 14). HSD caused a marked and significant elevation in coronary blood flow too (Figure 15). HSD infusion

significantly increased the LVD and myocardial contractility until 60 min of the postinfusion period (Figures 16 and 17).

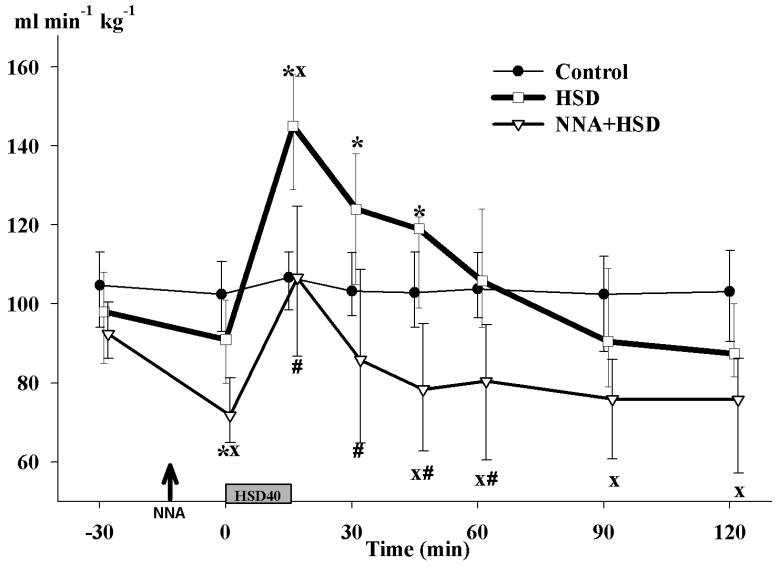

Figure 13. Changes in CI in the saline-treated control group (Group 4; circles), the HSD-treated group (squares) and the NNA+HSD treated group (triangles). * $P < 0.05$ within group; ^{X} $P < 0.05$ between groups *vs* saline-treated control group values; $^{\#}$ $P < 0.05$ between groups *vs* HSD-treated group values.

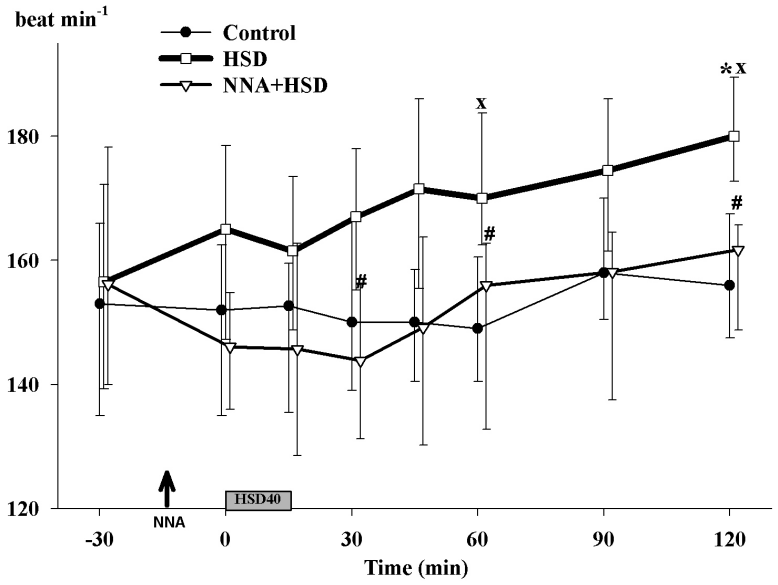

Figure 14. Changes in HR in the saline-treated control group (Group 4; circles), the HSD-treated group (squares) and the NNA+HSD treated group (triangles). * $P < 0.05$ within group; ^{X} $P < 0.05$ between groups *vs* saline-treated control group values; $^{\#}$ $P < 0.05$ between groups *vs* HSD-treated group values.

The NNA pretreatment immediately decreased the CI before HSD infusion and significantly inhibited the HSD-induced CI elevation for 60 min (Figure 13). This treatment resulted in a lower HR as compared with the HSD infusion, especially in the postinfusion phase (Figure 14). Nonselective NOS inhibition decreased the HSD-induced coronary flow elevation (Figure 15). The preload index LVD did not differ significantly from the result in the HSD-only group, but the late effect of the NNA treatment was a noteworthy decrease as a percentage of LVD (Figure 16).

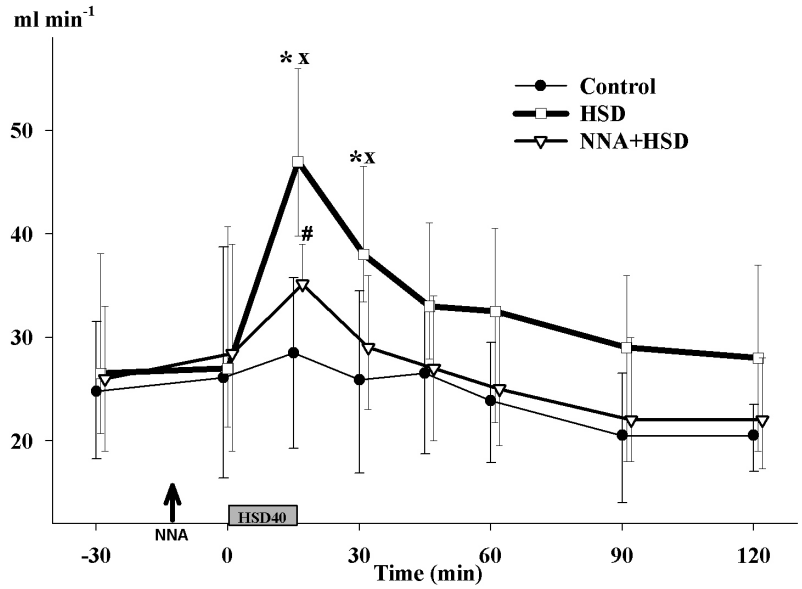

Figure 15. Changes in coronary flow in the saline-treated control group (Group 4; circles), the HSD-treated group (squares) and the NNA+HSD-treated group (triangles). * $P < 0.05$ within group; ^{X} $P < 0.05$ between groups *vs* saline-treated control group values; $^{\#}$ $P < 0.05$ between groups *vs* HSD-treated group values.

The cardiac contractility showed a biphasic reaction under this experimental protocol: NNA treatment immediately increased the end-systolic pressure-diameter relationship before volume loading, but the HSD-induced positive inotropy was decreased significantly until 60 min after HSD infusion and gradually increased again in the late phase of the postinfusion period (Figure 17).

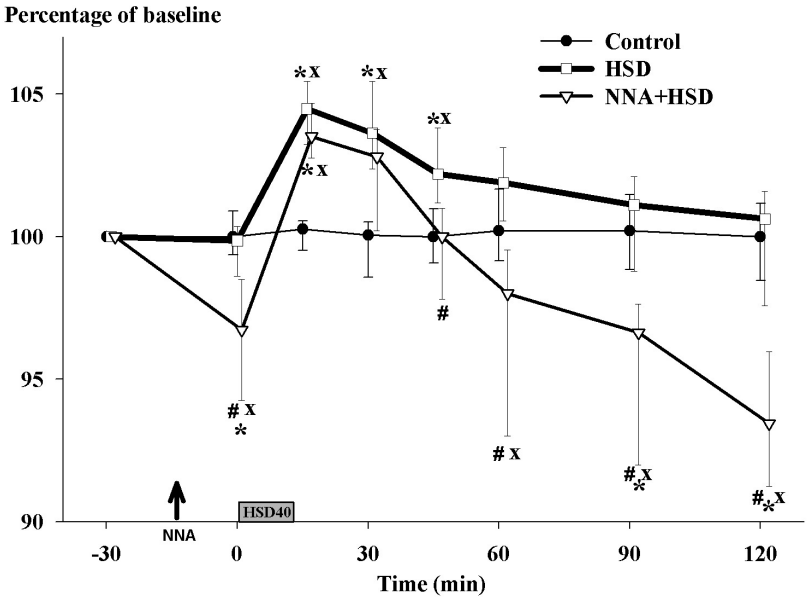

Figure 16. Changes in diastolic LVD in the saline-treated control group (Group 4; circles), the HSD-treated group (squares) and the NNA+HSD-treated group (open triangles).
* $P < 0.05$ within group; ^{X} $P < 0.05$ between groups *vs* saline-treated control group values;
$^{\#}$ $P < 0.05$ between groups *vs* HSD-treated group values.

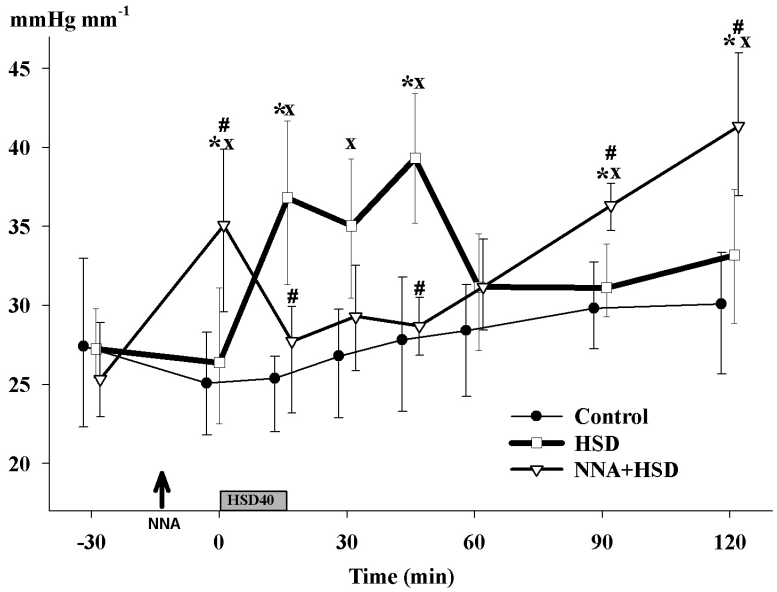

Figure 17. Changes in myocardial contractility in the saline-treated control group (Group 4; circles), the HSD-treated group (squares) and the NNA+HSD-treated group (triangles). * $P < 0.05$ within group; ^{X} $P < 0.05$ between groups *vs* saline-treated control group values; $^{\#}$ $P < 0.05$ between groups *vs* HSD-treated group values.

Biochemical changes in blood and myocardial tissue

The plasma ET-1 concentration was significantly increased (approximately 1.5-fold) by the end of the infusion (HSD group: 3.09±0.21 *vs* control Group 4: 1.72±0.11 fmol ml^{-1}), and remained significantly higher than in the control Group 4 up to the end of the 120-min observation period (Figure 18). Concomitantly, the HSD infusion caused a transient and significant, 45-min elevation in plasma NO_x level (Figure 19). The NNA pretreatment significantly enhanced the HSD-induced increase in plasma ET-1 level throughout the whole observation period as compared with the HSD-only group (Figure 18), while a lowered level of the plasma NO_x was detected in the course of the postinfusion period (Figure 19).

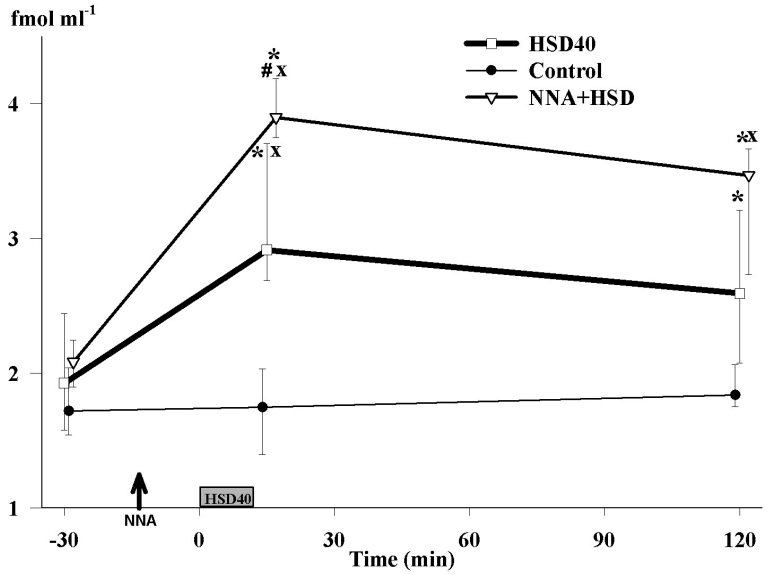

Figure 18. Changes in plasma ET-1 level in the saline-treated control group (Group 4; circles), the HSD-treated group (squares) and the NNA+HSD treated group (triangles). * $P < 0.05$ within group; ^{X} $P < 0.05$ between groups *vs* saline-treated control group values; $^{\#}$ $P < 0.05$ between groups *vs* HSD-treated group values.

However, 120 min after the HSD infusion, the myocardial cNOS activity was significantly lower as compared with that in the control Group 4 (Figure 20A). In these biopsies, the tissue MPO activity was significantly increased relative to the control Group 4 (Figure 20B). The nonselective NOS inhibitor pretreatment lowered the HSD-induced decrease in myocardial cNOS activity more appreciable by the end of the postinfusion period, but the NOS values were not significantly different from those in the HSD group (Figure 20A). The administration of NNA increased the myocardial MPO activity, but the differences were not statisticaly significant in MPO activity between the HSD-only group and the NNA-pretreated groups.

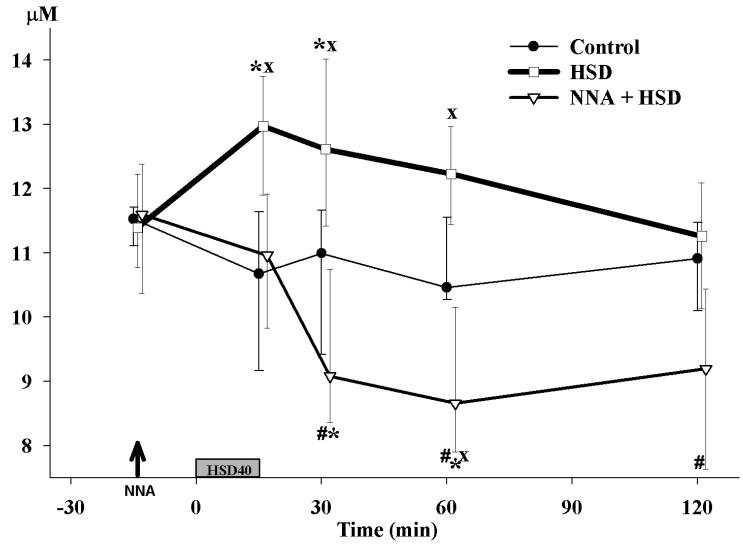

Figure 19. Changes in plasma NO_x level in the saline-treated control group (Group 4; circles), the HSD-treated group (squares) anf the NNA+HSD treated group (triangles). * $P < 0.05$ within group; ^{X} $P < 0.05$ between groups *vs* saline-treated control group values; $^{\#}$ $P < 0.05$ between groups *vs* HSD-treated group values.

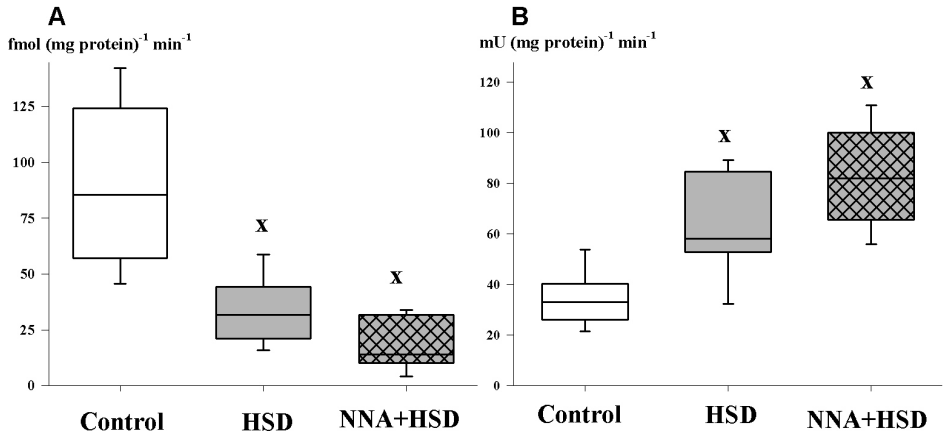

Figure 20. cNOS activities (A) and myocardial MPO activities (B) in myocardial tissue 120 min after treatment in the saline-treated (empty box), the HSD-treated (gray box) and the NNA-treated (checked box) animals. ^{X} $P < 0.05$ between groups *vs* saline-treated control group values. $^{\#}$ $P < 0.05$ between groups *vs* HSD-treated group values.

5. DISCUSSION

5.1. Discussion of Study I

Study I was designed to explore the connection between basal NO synthesis and the myocardial function in the unstressed dog, and we have shown that aspecific NOS inhibition leads to an increased myocardial contractility in this setting. However, it also became evident that a single effect (*i.e.* a NO deficiency-induced contractile response) involved several dependent variables. The results revealed that the lack of NO is accompanied by significant ET-1 release. Furthermore, a myocardial XOR activity elevation is also involved in the consequences of nonspecific NOS inhibition, and this reaction likewise plays an important modulator role in this process. These experimental data therefore suggest a suppressive, regulatory role for endogenous NO: it restrains or counteracts these mechanisms, which would otherwise increase the cardiac contractility.

Hemodynamic effects of NOS inhibition

Our study has demonstrated the efficacy of NNA-induced NOS inhibition via a decreased plasma NO_x level, together with prolonged increases in MAP and TPR and decreased CI and HR. NOS inhibitor treatment caused a decrease in the LV diastolic-systolic diameter difference, which points to an attenuation of the Frank-Starling response and might explain the decrease in CI (Prendergast 1997).

It has been evidenced that NO also influences HR by directly affecting the cardiac pacemaker activity, baroreflex transmission or the activity of the autonomic nervous system through enhancement of the bradycardic effects of vagal efferent (Balligand 1993, Conlon 1998). On the one hand, NO donors can inhibit the peripheral sympathetic neurotransmission by presynaptic reduction of the release of noradrenaline (Schwarz 1995, Choate 1999). On the other hand, the oral intake of an NOS inhibitor enhances baroreceptor reflex gain with decreased HR (Vasquez 1994). As a result of the multilevel HR regulation, diminished NO production potentiates the reflex bradycardia.

In our research protocol, the LV end-systolic pressure-diameter relationship was recorded as a preload-independent index of cardiac contractility (Goldfarb 1990). Consistent with our earlier results, NNA treatment caused a significant increase in heart contractility (Kaszaki 1996). Indeed, a detailed analysis of our experimental data suggested that the mechanism of the NNA-induced myocardial contractility elevation is a complex process which involves several variables (see explanation below).

NO effects on cardiac contractility

Previous studies have demonstrated that high concentrations of endogenous NO can produce a negative inotropic effect (Brady 1993, Brunner 2001), primarily due to a reduction in myofilament responsiveness to Ca^{2+} (Brunner 2001). However, other groups have reported that the stimulation of myocardial NO production can offset the increase in contraction in response to a rise in intracellular Ca^{2+} (Kojda 1996, Casadei 2003).

Experiments with NO donors have often shown the opposing effects of NO on the myocardial contraction and HR. It has been revealed that the reaction is biphasic and dose-dependent, *i.e.* low concentrations of NO donors (0.1-10 mM) increase the contractility and HR, whereas higher concentrations (> 100 mM) elicit negative inotropic and chronotropic effects (Casadei 2003, Massion 2003, Gonzalez 2008).

Recent evidence has clearly demonstrated the critical role of NOS isoforms in the regulation of cardiac contractility: in nNOS-null mice, the inotropic response was suppressed, whereas in eNOS-deficient mice, the contractility was enhanced (Barouch 2002). Moreover, there is a spatial confinement of NO signaling in the heart. Specifically, in the sarcoplasmic reticulum, nNOS colocalizes with the ryanodine receptor, and the activation of nNOS positively modulates the cardiac contractility via the augmentation of Ca^{2+} release in response to frequency and β-adrenergic receptor stimulation. In contrast, the eNOS isoform is coupled to the β_3-adrenergic receptor and inhibits L-type Ca^{2+} channels, accordingly inhibiting β-adrenergic receptor-mediated increases in myocardial contractility (Barouch 2002, Casadei 2003).

Taken together, these findings indicate that, unlike eNOS, the cardiac nNOS isoform is an important physiological determinant of basal contractility in the mammalian myocardium, which suggests that nNOS-derived NO may exert a negative feedback control on Ca^{2+} entry, since an increase in intracellular Ca^{2+} would stimulate the nNOS synthesis of NO, which in turn would inhibit Ca^{2+} influx through the L-type Ca^{2+} channels (Casadei 2003).

In light of these results, nonspecific NOS inhibition ignores the specialization and spatial confinement of signaling of the cardiac NOS isoforms, and effectively (in higher doses presumably more effectively) abrogates the modulator effect of the predominant NOS isoform.

An important consequence of NOS inhibition could be an imbalance in the positive-negative inotropy (vasoconstrictor-vasodilator) relationship, leading primarily to ET-1 release. Since NO may normally moderate ET-1 production and action (Boulanger 1990, Kourembanas 1993), the inhibition of NO synthesis can result in increased plasma levels of

ET-1 (Richard 1995, Filep 1997). It has been demonstrated that, after acute NOS blockade, the predominant pressor mechanism is associated with a marked increase in ET-A/ET-B receptor activation rather than with increases in alpha-1, angiotensin 1 or vasopressin V1/V2 receptor activation (Banting 1996).

As far as we are aware, this is the first *in vivo* evidence of positive inotropy caused by ET-1 following NOS inhibition. Several aspects of the model highlight that the NNA-induced circulatory responses are characterized by predominantly ET-related immediate hemodynamic changes, demonstrating that this peptide is an important determinant of the increase in cardiac contractility. This observation supports the *in vitro* finding that NOS inhibition enhances the inotropic response to ET-1 (Kinnunen 2000). Exogenous ET-1 infusion may increase the *in vivo* cardiac contractility significantly, an effect that can be prevented by ET-A receptor antagonist pretreatment (Konrad 2005). In our study, the plasma ET-1 level gradually increased up to the end of the experiments, which may suggest that, after induction by NNA treatment, it was continuously replaced from the cellular sources. The results also revealed that the ET-1 peptide induced positive inotropy through the activation of ET-A receptors.

Recent evidence strongly suggests that the target area of NO and/or ET-1 is the same cellular microdomain for the modulation of cardiomyocyte contractility, via regulation of the L-type Ca^{2+} channels, but in the opposite sense (Casadei 2003, Robu 2003).

Effects of ET-A receptor antagonism

ET-A receptor antagonism by the ETR-P1/fl peptide proved effective in reducing the signs of vasoconstriction. As a result, the CI displayed an immediate significant increase and did not decline below the control level following NNA treatment, while the NOS inhibition-induced elevation in TPR was lowered. Pretreatment with the ET-A receptor antagonist significantly elevated the plasma level of NO_x. This observation is in agreement with the data of Maeda *et al.*, who demonstrated that an exercise-induced increase in ET-1 significantly lowered the plasma NO_x concentration in the kidney, whereas pretreatment with an ET-A receptor antagonist resulted in significantly higher NO production (Maeda 2004). This elevation in NO production, presumably through activated ET-B receptor-induced NO release (Verhaar 1998), has a role in normalizing the LV diastolic-systolic diameter difference. Additionally, the plasma level of ET-1 was significantly lower and in this case the positive inotropic effect was missing. Hence, the volume per cardiac cycle increased, which maintained sufficient perfusion.

The ETR-p1/fl peptide, which was used in our study, has a special feature: it is an intramolecular complementary peptide of the ET-A receptors; as such, it can specifically

recognize and bind the circulating ET-1 molecules (Baranyi 1995, Baranyi 1998). It has been shown that ETR-p1/fl peptide infusion in the same dose induces significant increases in CI and LV systolic-diastolic difference and significant decreases in TPR and plasma ET-1 level, while it does not influence the MAP, HR and cardiac contractility (Kaszaki 1997).

From the above results, it can be concluded that a deficit of ET-1 does not cause notable changes in macrohemodynamic parameters. Under normal, unstressed conditions, the regulatory effect of ET-1 could predominate at the level of the microcirculation. In contrast with the lack of ET-1, under conditions involving an NO shortage, both micro- and macrocirculatory disturbances and alterations in cardiac function can be observed, in which a significant role could be played by the elevated ET-1 level.

Importance of interaction of myocardial nNOS and XOR

In our study, NOS inhibition resulted in an increase in myocardial XOR activity. There is evidence that the activity of XOR, a major source of the production of reactive oxygen intermediates in the heart, is increased in the LV myocardium in nNOS-deficient mice (Khan 2004, Kinugawa 2005). Kinugawa *et al.* demonstrated spatially confined interactions between nNOS and XOR (Kinugawa 2005). These enzymes co-immunoprecipitate and colocalize in the sarcoplasmic reticulum of the cardiomyocytes, suggesting that nNOS gene deletion may have wider implications on the myocardial redox state. Additionally, NO has a direct inhibitory effect on XOR activity *in vitro* (Hassoun 1995). Hence, under normal circumstances, nNOS-derived NO inhibits XOR activity in a paracrine manner through the direct binding of NO to the iron-sulfur moiety of the enzyme (Hassoun 1995, Khan 2004). A deficiency of nNOS (but not eNOS) leads to a marked increase in XOR-mediated superoxide production and may contribute to a further reduction of the bioavailability of eNOS-derived NO in the myocardium, which in turn depresses myocardial excitation-contraction coupling (Khan 2004).

Indeed, many studies have shown that the superoxide radical produced by XOR decreases the myocardial contractility in a variety of cardiovascular pathologies. On the other hand, it has also been shown that the positive inotropic effect of ET-1 is mediated by mitochondrial reactive oxygen intermediates (Channon 2002, De Giusti 2008). Furthermore, ET-1 significantly increases intracellular superoxide levels in isolated normal cardiac myocytes (Caldiz 2007). It is important to note that XOR mimicked the effect of ET-1 in this system and, additionally, the effect of ET-1 was abolished by specific ET-A receptor antagonist treatment (Zeng 2008). Collectively, these *in vitro* observations, together with our *in vivo* data, suggest that ET-1 induces contractility changes via the stimulation of superoxide

generation. Given the strong positive inotropic effects of ET-1, it will be important to determine how much of the NNA-induced contractility change is due to ET-1 and the extent to which it is influenced by superoxide radical production in normal cardiac tissue.

It should be noted that direct and indirect (or peripheral and central) effects of NOS and ET antagonism are difficult to distinguish *in vivo*. Taken together, the increase in LV contractility could be due to several factors, including increases in preload and coronary blood flow or the effects of reflex autonomic changes (*e.g.* baroreceptor responses). However, the results underline the significance of diminished NO production in myocardial XOR activity changes and ET-1 release, and these processes could contribute indirectly to the alterations in cardiac contractility through ET-A receptor activation.

In conclusion, secondary reactions linked to reduced NO production play important, and perhaps crucial roles in shaping NO-induced contractility changes in the normal myocardium. We propose that this pathway represents a homeostatic mechanism by which a mismatch between NO supply and demand in the cardiac muscle is translated into an increase in contractility.

5.2. Discussion of Study II

This study was designed to explore a connection between the increased peripheral flow-induced changes and cardiac contractility alterations. Another aim was to outline the role of NO in the HSD-evoked hemodynamic changes and to investigate the reliability of small-volume resuscitation in conditions associated with diminished NO production.

The results revealed that generated vasoactive mediators are involved in HSD-induced circulatory changes and in alterations of cardiac contractility through NO-dependent and independent mechanisms, and suggest the preponderance of ET-1 in this process. Furthermore, it can be concluded, that HSD is less effective in NO-deficient states.

Hemodynamic effects of HSD

HSD solutions bring about rapid changes in the macro- and microhemodynamics in various circulatory beds. This could be related to a number of mechanisms, including the redistribution of fluid from the interstitium to the intravascular space; a direct relaxing action on the vascular smooth muscle; a blood fluidity improvement by hemodilution; and a stretch-induced ventricular dilatation caused by a pressure or volume overload (Janicki 2006). In our experiments, the molecular weight of the dextran component of the HSD solution was 40 kD. *In vivo*, solutions of 40 kD dextran are advantageous when an improvement in blood flow is specifically required, while a higher molecular weight dextran (e.g. 70 kD) is preferable when

a longer circulation time and a plasma volume expansion are the primary goals. The rationale of our approach was to achieve a rapid increase in flow velocity. Indeed, the HSD infusion resulted in prompt increases in MAP and CO and a decrease in TPR; the HR and coronary flow increased concomitantly, and a significant rise in myocardial contractility was noted in the early phase of the postinfusion period.

An expansion of the blood volume has been shown to increase the HR and blood pressure via the sympathetic nerves in a number of species, and this effect is based primarily on the activation of volume receptors at the venous-atrial junctions of the heart (Coote 2005). Stimulation of volume receptors by acute volume expansion results in an elevation of the plasma atrial natriuretic factor level, which correlates significantly with the right atrial pressure. An increase in atrial natriuretic factor level contributes to the elevation in preload, which could indirectly influence the LV contractility (Nakajima 2005). Blockade of the autonomic nervous system (by sinoaortic denervation, vagotomy or hexamethonium treatment) did not influence the increase in plasma atrial natriuretic factor level during volume expansion by 20 ml/kg dextran in lactated Ringer's solution (Kohara 1989, Otsuka 1989).

The increased LV preload might contribute to the positive inotropy, but the mechanism of the HSD-induced myocardial contractility elevation is rather complex, and this change can not be a simple consequence of an increased intravascular volume. This hemodynamic pattern could increase the coronary blood flow too (Gregg effect), which itself could lead to positive inotropy (Gregg 1963, Feigl 1983).

Several studies suggest the increased activity of the humoral positive inotropic agents in the case of volume expansion. Wade *et al.* and Elgjo *et al.* demonstrated elevations in plasma renin and norepinephrine following HSD or hypertonic saline infusion (Wade 1991, Elgjo 1994). Angiotensin II activation through AT1 receptors potentiates Na^+/H^+ exchange activity, and an increase in intraneuronal Na^+ will lead to the excessive release of norepinephrine from the sympathetic neurons (Mackins 2006). Moreover, in human patients treated with the angiotensin convertase enzyme inhibitor quinapril, the plasma norepinephrine level did not change during a volume overload. This was not observed in the case of untreated patients (Volpe 1992).

It has been shown that an acute volume expansion caused by a rapid infusion of hypertonic colloid solution results in an increase in plasma ET-1 (Boldt 1994). Exogenous ET-1 infusion may increase the *in vivo* cardiac contractility significantly, an effect that can be prevented by ET-A receptor antagonist pretreatment (Konrad 2005). The results revealed that the plasma level of ET-1 increased significantly in this setup too, and the peptide induced

positive inotropy through the activation of ET-A receptors (Eszlári 2008, Czóbel 2009). The plasma ET-1 level subsequently remained elevated until the end of the experiment, which may suggest that, after induction by HSD, it was continuously replaced from the endothelium or myocardial cells. Accordingly, it seems that HSD-induced mechanical stimuli result in an elevated protease output in the plasma (Mackins 2006), which can increase the formation of ET-1 from pre-pro ET or from big-ET (an elevated flux of proteases could be increase level of ET-1 preformed from its cellular sources).

The postinfusion period was characterized by significant rises in the plasma NO and ET-1 concentrations. NO and ET-1 release or synthesis are triggered by various mechanical stimuli, such as increased osmolarity due to by cell shrinkage (Schramek 1993), fluid shear stress on the endothelium (Kuchan 1993, Fissthaler 2000) and the physical stretching of smooth muscle vascular cells or cardiomyocytes (van Wamel 2001, Casadei 2003).

In our experiments, the volume expansion effected by the HSD infusion resulted in a moderate elevation of the plasma level of NO_x, an end-product of NO, which could contribute positively to the LV contractility in this concentration. Although previous studies have indicated that excessive NO delivery from inflammatory cells (or cytokine-stimulated cardiomyocytes themselves) may result in profound cellular disturbances leading to attenuated cardiac contractility (Massion 2003), others have reported that the stimulation of myocardial NO production can offset the increase in contraction in response to a rise in intracellular Ca^{2+}. Cardiac NO production is also activated by stretching and, under these conditions, NO has been shown to facilitate the Frank-Starling response and to contribute to the increase in intracellular Ca^{2+} transients that mediates the slow increase in contraction in response to stretching (Casadei 2003).

In the later phase of the postinfusion period**,** the HR gradually increased, the CI decreased and the myocardial contractility returned to a near-baseline level, while the TPR increased significantly. Further, these hemodynamic and biochemical changes were accompanied by enhanced myocardial MPO activity and decreases in myocardial cNOS activity. It has been proved in a number of diseases involving circulatory failure (myocardial infarction, cardiogenic shock, atherosclerosis and congestive heart failure) and also in surgical interventions that an increased ET-1 level is associated with decreased NO production (Warner 1999, Ovadia 2002). This marked decrease in NOS activity may be an indirect consequence of the elevation in ET-1 level. One possible explanation of this phenomenon is that the HSD-induced ET-1 release may be attributed to an increase in superoxide radical production, simultaneously with significant NO production, and this leads to the formation of

peroxynitrite, a known inhibitor of NOS activity (Sheehy 1998, Ovadia 2002). Furthermore, ET-1 *per se* could inhibit endogenous NO synthesis through enhanced asymmetric dimethylarginine synthesis and ET-A receptor activation (Ohnishi 2002). On the other hand, peroxynitrite-mediated myocardial protein nitration has been associated with a depressed cardiac pump function. Borbely *et al.* proposed that alpha-actinin is a target for peroxynitrite in the human myocardium; and its nitration can induce a contractile dysfunction (Borbely 2005). Additionally, an *in vivo* interaction might occur between the increased nitrite level and myocardial MPO, affording reactive nitrosyl derivatives (Cooper 1992) and leading to protein nitration too.

Effects of NNA pretreatment

Since the role of NO in regulating the cardiac function is complex and controversial, it was important to determine what proportions of the HSD-induced circulatory and myocardial contractility change were due to NO-dependent and to NO-independent mechanisms. Our findings with NNA pretreatment indicate that the HSD-induced hemodynamic changes were mediated mainly through NO-dependent mechanism.

NNA pretreatment definitely inhibited the HSD-evoked favorable hemodynamic features, the CI declined below the control level, and TPR increased enormously during the observation period. HSD-induced NO_x release was blocked by NNA pretreatment at 15 min of the postinfusion period. Moreover, the plasma level of ET-1 was significantly higher in the NNA+HSD group than in the HSD-only group. The extreme elevation of the ET-1 level could explain the increased TPR and venoconstriction, but ET-1-related positive chronotropy was not detected. As a result of NNA pretreatment, the HSD-induced HR elevation failed; it remained permanently at the control level. Our results are consistent with findings of the acute study by Pontieri *et al.*, in which microinjections of a nonselective NOS inhibitor into the nucleus tractus solitarius increased the baroreceptor reflex gain with decreased HR in conscious normotensive rats (Pontieri 1998). In another study, the oral intake of an NOS inhibitor enhanced the baroreceptor reflex gain due to a potentiation of reflex bradycardia (Vasquez 1994). Inhibition of eNOS activity in the the nucleus tractus solitarius may increase the baroreceptor reflex gain via disinhibition (Waki 2003). Overall, it seems that HR regulation under volume expansion is an NO-dependent rather than an ET-1-dependent process.

NNA pretreatment did not influence the HSD-induced increase in LVD during the acute phase of volume expansion. Though NO largely accounts for the myocardial diastolic properties, the HSD-induced LVD increase seems to be NO-independent. This could be

explained by the mechanical atrial stretch due to volume expansion, or by the high sodium concentration of the HSD infusion, which stimulates atrial natriuretic peptide release with an enhanced ventricular diastole (Arjamaa 1985). However, in the late phase of the postinfusion period, the effect of NNA pretreatment was characterized by a gradually decreased LVD with a lowered filling volume, because of the ET-1-induced venoconstriction.

Cardiac contractility is essentially dependent on oxygen supply and is characterized by an extremely high oxygen consumption. An HSD-induced volume expansion demands a higher energy supply, which is covered by an increased coronary flow. It has been evidenced that positive inotropy related to the increased utilization of ATP leading to the release of adenine nucleosides. Moreover, it has been demonstrated that exogenous ET-1 causes a significant elevation in the purine metabolism and stimulates adenosine release (Zima 2002). However, the inhibition of NO production reversed the cardiac contractility changes, with an extreme increase in plasma ET-1 level, which could be responsible for the decreased coronary perfusion (Juhász-Nagy 1999). Hence, the volume per cardiac cycle decreased to a level which was not able to maintain sufficient tissue perfusion. Accordingly, the diminished NO production with enhanced ET-1 release as an unfavorable side-effect of HSD fluid therapy could have a significant role in a cardiac power deficit. In the later phase of the postinfusion period, the coronary perfusion did not decrease under the control level, despite the decreased cardiac NO production. This could be explained by the enhanced level of adenosine originating from ATP breakdown, which is the main mediator of coronary metabolic adaptation in the heart (Kékesi 2002). It could be assumed that the gradual increase in cardiac contractility at the end of the observation period was due to the adapted cardiac perfusion with the energy supply. This late positive inotropy means that volume expansion can stimulate the release of other vasoconstrictors, and not only ET-1. In this regard, the roles of the renin-angiotensin system and catecholamines could be of interest (Mackins 2006). Overall, the HSD-induced positive inotropy proved an indirect NO-dependent process, which predominates through the coronary perfusion rather than through direct effects on cardiac contractility.

The data reported here demonstrate that HSD-induced mechanical stimuli cause significant peripheral NO and ET-1 release in the heart. This response may lead to an upset of the ET-1 - NO balance, an unfavorable side-effect of HSD fluid therapy. As a result of NNA pretreatment, the ET-1 - NO balance is disturbed mainly in the late phase of the postinfusion period, since the activity of NOS is decreased. The maintained NO production is absolutely necessary for normal peripheral and cardiac hemodynamic functions. Our results suggest that

medication via HSD volume therapy could exert a negative influence on the outcome in numerous diseases associated with a decreased NO production (myocardial infarction, congestive heart failure, atherosclerosis and diabetes).

6. SUMMARY OF NEW FINDINGS

NO plays a vital role in controlling the peripheral circulation and myocardial contractility, and its lack could modify the circulatory regulation to a great extent. Our experimental data suggest a suppressive, regulatory role for endogenous NO: it restrains or counteracts several mechanisms, which would otherwise increase the cardiac contractility. The bioavailability of NO or its amount relative to other, vasoregulator, inotropic agents is of crucial importance.

I. A diminished NO production leads to secondary reactions by which a mismatch between NO supply and demand in the cardiac muscle is translated into an increase in cardiac contractility. A reduced NO synthesis leads to an increased superoxide production, a myocardial XOR activity elevation and preponderant vasoconstrictor effects, which decrease the CO and increase the myocardial contractility through an ET-A receptor-dependent mechanism.

II. HSD-induced mechanical stimuli cause significant peripheral NO and vasoconstrictor mediator release, and this response may lead to an upset of the NO – inotropic mediator balance. This is an unfavorable side-effect of HSD fluid therapy.

III. The positive inotropy is an indirect consequence of HSD administration. This NO-dependent process predominates through coronary perfusion changes.

IV. Volume therapy with HSD creates a situation which is unfavorable from a hemodynamic aspect and could exert a negative influence on the outcome of circulatory conditions associated with a decreased NO production.

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9. ANNEX

1. Czóbel M, Kaszaki J, **Molnár G**, Nagy S, Boros M: Nonspecific inhibition of nitric oxide synthesis evokes endothelin-dependent increases in myocardial contractility. Nitric Oxide Biol Chem 21: 201-209, 2009.
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