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Nonspecific inhibition of nitric oxide synthesis evokes endothelin-dependent increases in myocardial contractility

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ABSTRACT

The role of endogenous nitric oxide (NO) in modulating myocardial contractility is still unclear, in part because of unknown, secondary effects of blocking NO release. We hypothesized that the nonspecific inhibition of nitric oxide synthase (NOS) enhances endothelin-1 (ET-1) effects, which can play a role in ET-A receptor-dependent myocardial contractile responses. The myocardial contractility was estimated from the slope of the left ventricular end-systolic pressure–diameter relationship in closed-chest, pentobarbital-anesthetized dogs. Group 1 ($n = 7$) was the saline-treated control, while in groups 2 ($n = 7$) and 3 ($n = 7$) *N*-nitro-L-arginine (NNA, 4 mg kg^{-1}), a nonselective NOS blocker, was administered with or without pretreatment with the ET-A receptor antagonist ETR-P1/fl peptide (100 nmol kg^{-1} iv). Plasma ET-1, nitrite/nitrate (NO_x) and blood superoxide levels were measured, and myocardial ET-1 content and xanthine oxidoreductase (XOR) activity were determined from myocardial biopsies. The infusion of NNA over 120 min decreased the plasma NO_x, significantly elevated the plasma ET-1 and blood superoxide levels, and in parallel greatly increased the left ventricular contractility as compared with the untreated controls [47.5 *vs* 30 mm Hg mm^{-1}]. The myocardial ET-1 content decreased simultaneously, while the XOR activity and blood superoxide level were significantly elevated. These effects, including NNA-induced positive inotropy, were significantly suppressed by pretreatment with ETR-P1/fl peptide. These results demonstrate that a diminished NO synthesis leads to a preponderant ET-1 effect, which increases myocardial contractility through an ET-A receptor-dependent mechanism.

Introduction

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, including neuronal NO synthase (nNOS or NOS1), inducible NOS (iNOS/NOS2) and endothelial NOS (eNOS/NOS3) [1]. Although NO is regarded as predominantly a peripheral vasoactive mediator, the mammalian heart also expresses all three isoforms of NOS, and NO has several specific effects on the normal myocardium too. These involve enhancement of myocardial relaxation and the diastolic function [2–4], an increase or decrease of β-adrenergic responses [5,6], modulation of the force–frequency relationship [7] and mediation of parasympathetic cholinergic effects [8].

A number of studies have shown that NO is released in the coronary artery system and influences myocardial contractility directly, but the exact role of endogenous NO production in the regulation of the cardiac contractility is still controversial [5,9–11], mainly because of the disproportionate extrapolation from results relating to modulation of the NO output. The consequences of an 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 [6,12,13]. 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 [10,14]. Similarly, studies with eNOS-deficient mice strains have yielded contradictory results [9,15]. While the positive inotropic response is enhanced in eNOS-deficient mice [9,16], this effect was not present when isolated cardiomyocytes from eNOS knock-out mice were used [17].

A possible explanation for these versatile 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 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. In this context, several lines of evidence point to the rather complex role of two independent, but potentially interconnected bioactive systems.

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Firstly, it has been shown that a constant NO release will normally suppress endothelin (ET) production and action [18,19]. ET-1 is one of the most powerful endogenous vasoconstrictor substances [20], and the pressor effect of NOS inhibition is paralleled by an elevation of plasma ET-1 [21,22]. The vasoconstrictive effects of ET-1 are mediated predominantly via the endothelin-A (ET-A) receptors present on the vascular smooth muscle cells. The ET-B receptors mediate vasoconstriction (ET-B_2) and vasodilation (ET-B_1), but ET-1 has a higher affinity for ET-A than for ET-B receptor subtypes [4,23].

Secondly, recent *in vitro* findings suggest that the positive inotropic effect of ET-1 may be mediated by an intracellular pathway triggered by mitochondrial superoxide formation [24]. Furthermore, it has been demonstrated that endothelial dysfunction is associated with reduced NO bioavailability and increased superoxide production in the cardiovascular system [25,26]. In line with this, there is important crosstalk between xanthine oxidoreductase (XOR), a prototypic superoxide-producing enzyme, and cardiac NO signaling. The XOR activity is elevated in the left ventricular myocardium of nNOS-deficient mice, leading to increased superoxide production, which again may play a role in contractility alteration [27,28].

Taken together, we hypothesized that the nonspecific NOS inhibition would unmask the positive inotropic effect of ET-1. Our goal was to devise a study design that helps establish a conceptual approach to a single cause – multiple effect phenomenon, and thereby identify a causal chain mechanism relevant to NO-linked contractility responses. To this end, we characterized the myocardial contractility 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).

Materials and methods

The experiments were performed in adherence to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Surgical procedure

The experiments were performed on 21 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, USA). 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 the experiments. A Swan-Ganz thermodilution catheter was positioned into the pulmonary artery via the right femoral vein to measure pulmonary artery pressure (PAP) and cardiac output (CO). An inflatable balloon-catheter 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, USA) was introduced into the left ventricle through the left internal carotid artery to monitor the left ventricular pressure (LVP). A left thoracotomy was performed and a pair of ultrasonic dimension crystals (3 MHz, ID-4, Custom Transducers, Poway, CA, USA) were sutured onto the anterior and posterior walls of the left ventricle, opposite each other, for measurement of the left ventricular 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. 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.

Hemodynamic measurements

All hemodynamic signals (pressures and left ventricular diameter) were registered with a computerized data-acquisition system (SPEL Advanced Haemosys 2.72, Experimetria Ltd., Budapest, Hungary). Mean arterial pressure, central venous pressure and pulmonary artery pressures were monitored with Statham P23Db transducers. The heart rate (HR) was calculated from the mean arterial pressure curve. The cardiac index (CI) was determined by thermodilution, using a Cardiostar CO-100 computer (Experimetria Ltd., Budapest, Hungary). 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, USA) via the left ventricular pressure and left ventricular diameter signals, the end-systolic elastance, as a parameter of the left ventricular myocardial contractility, was estimated from the slope of the end-systolic pressure–diameter relationship [29] with a computer program [13]. 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–diameter relationship, and the variance of fitting was determined. The calculation was based on a minimum of eight cardiac cycles.

Plasma and cardiac tissue ET-1 measurements

Two milliliters of blood samples were drawn from the jugular vein into chilled polypropylene tubes containing EDTA (1 mg ml^{-1}) and aprotinin (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 1200*g* for 10 min at 4 °C. The plasma samples were then collected and stored at −70 °C until assay.

For tissue samples, full-thickness heart biopsies were homogenized in phosphate buffer and the homogenate was centrifuged at 4 °C for 30 min at 24,000*g*. The supernatants and 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 [30].

Whole blood superoxide production

Superoxide production was assessed by the lucigenin-enhanced chemiluminescence assay of Zimmermann et al. [31]. Briefly, 10 μl blood samples were added to 1 ml Hank's balanced solution (without phenol red, PAA Cell Culture Company) at 37 °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).

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Determination of myocardial 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 °C for 20 min at 24,000*g* 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 (XO activity) of the electron acceptor methylene blue [32].

Experimental protocol

The animals were randomly allotted 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, USA) 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 (Kurabo Ltd., Osaka, Japan) [33,34] 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.

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, median values and 75th and 25th percentiles are given. *P*-Values <0.05 were considered significant.

Results

In the control group, 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^{-1} NNA resulted in sustained increases in mean arterial pressure (Fig. 1A) and CVP (data not shown). Total peripheral vascular resistance was increased nearly 2-fold as compared with the baseline values 105 min after NNA treatment (Fig. 1C).

Nonspecific NOS inhibition caused an approximately 25% decrease in cardiac index (Fig. 1B), and a significant increase in myocardial contractility up to the end of the observation period (Fig. 2A). Heart rate decreased gradually and the difference between the left ventricular diastolic and systolic diameters (as a percentage of the baseline) was also reduced significantly (Table 1).

The ETR-P1/fl peptide pretreatment mitigated the NNA-induced mean arterial pressure and CVP elevations, but the differences between the values for the NNA and ETR-P1/fl peptide + NNA groups were statistically not significant (Fig. 1A). The pretreatment effectively antagonized the NOS inhibition-induced total peripheral vascular resistance elevation in the first 60 min after NNA administration (Fig. 1C).

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 cardiac index decrease was then significantly reduced (Fig. 1B). The pretreatment mitigated the NNA-induced decrease in heart rate, but the difference between the NNA and ETR-P1/fl peptide + NNA groups was statistically not significant (Table 1). However, ETR-P1/fl peptide pretreatment significantly inhibited the NNA-induced decrease in the left ventricular diastolic–systolic diameter difference (Table 1) and the NNA-induced elevation in myocardial contractility (Fig. 2A).

Blood biochemical changes

The plasma ET-1 concentration gradually rose to approximately 1.5-fold following NNA infusion and remained significantly higher than in the control group up to 120 min in the observation period (Fig. 2B). The NOS inhibitor treatment caused a significant decrease in plasma NO_x level at 90 min (Fig. 3A). Concomitantly, an approximately 5-fold increase in blood superoxide production was observed (Fig. 3B).

The ETR-P1/fl peptide pretreatment prevented the NNA-induced increase in plasma ET-1 level throughout the observation period (Fig. 2B) and significantly inhibited the NNA-induced superoxide production in the blood (Fig. 3B). 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 (Fig. 3A).

Myocardial tissue

One hundred thirty-five minutes after NNA treatment, the myocardial ET-1 content was significantly decreased, while the tissue XOR activity was significantly elevated as compared with the control group (Fig. 4A and B).

The ET-A antagonist pretreatment prevented the NNA-induced changes in myocardial ET-1 content and XOR activity 135 min after NNA infusion (Fig. 4A and B).

Discussion

This study was designed to explore the connection between basal NO synthesis and 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 and a simultaneous ET-1 decrease in the cardiac tissue. Furthermore, 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 the NNA-induced NOS inhibition via the decreased plasma NO_x level, together with prolonged increases in mean arterial pressure and total peripheral vascular resistance, and decreased CI and heart rate. NOS inhibitor

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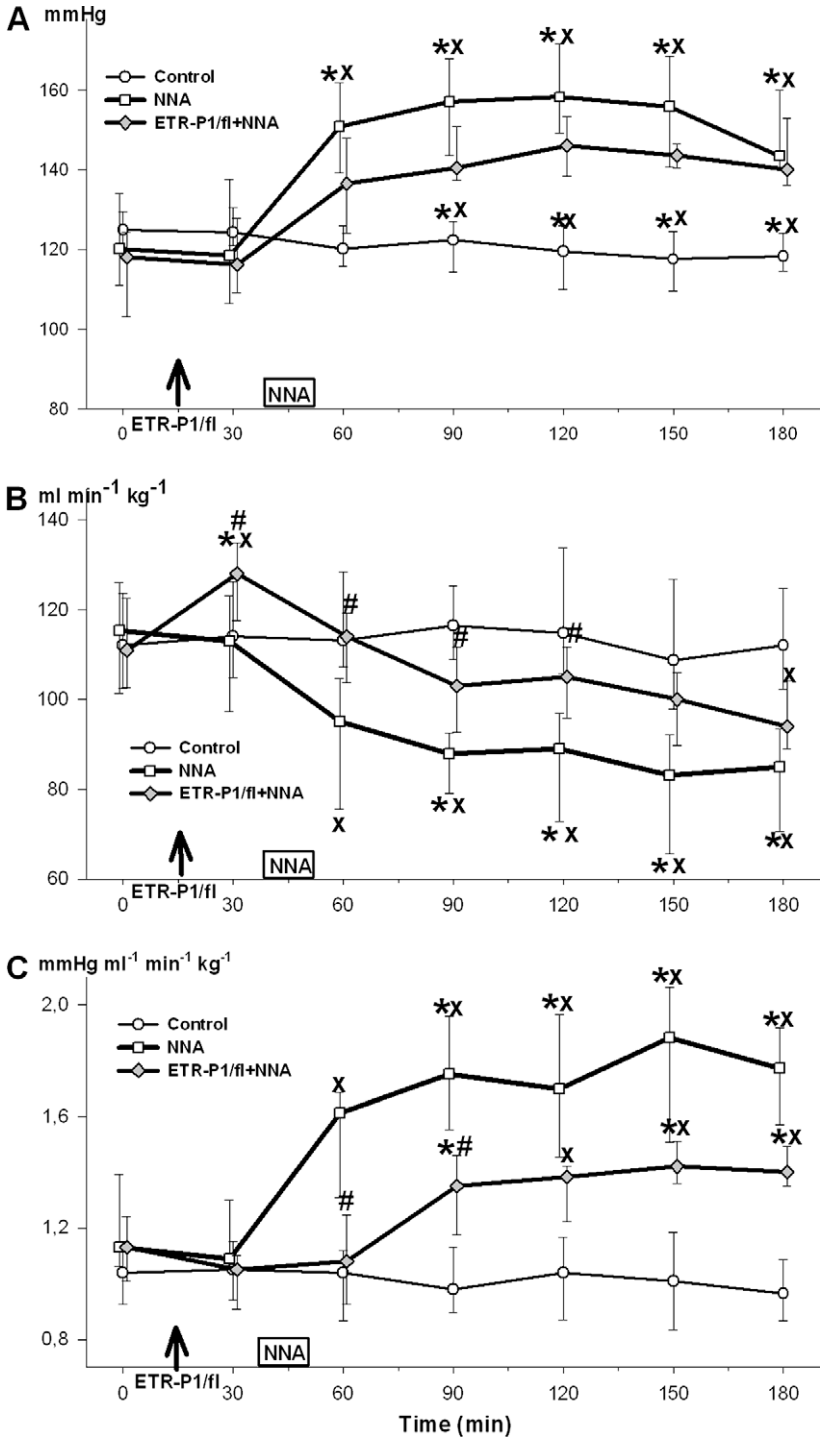

Fig. 1. Changes in mean arterial pressure (A), cardiac index (B) and total peripheral resistance (C) in the control group (black circles), the NNA-treated group (open squares) and the ETR-P1/fl + NNA-treated group (gray diamonds). Data are expressed as medians ± 25th and 75th percentiles. $^{*}P < 0.05$ within group; $^{X}P < 0.05$ between groups *vs* saline-treated control group values, $^{\#}P < 0.05$ between groups *vs* NNA-treated group values.

treatment caused a decrease in the left ventricular diastolic–systolic diameter difference, which points to an attenuation of the Frank–Starling response and might explain the decrease in cardiac index [35].

In our research protocol, the left ventricular end-systolic pressure–diameter relationship was recorded as a preload-independent index of cardiac contractility [29]. Consistent with our earlier results, NNA treatment caused a significant increase in heart contractility [13]. 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 [10,15], primarily due to a reduction in myofilament responsiveness to

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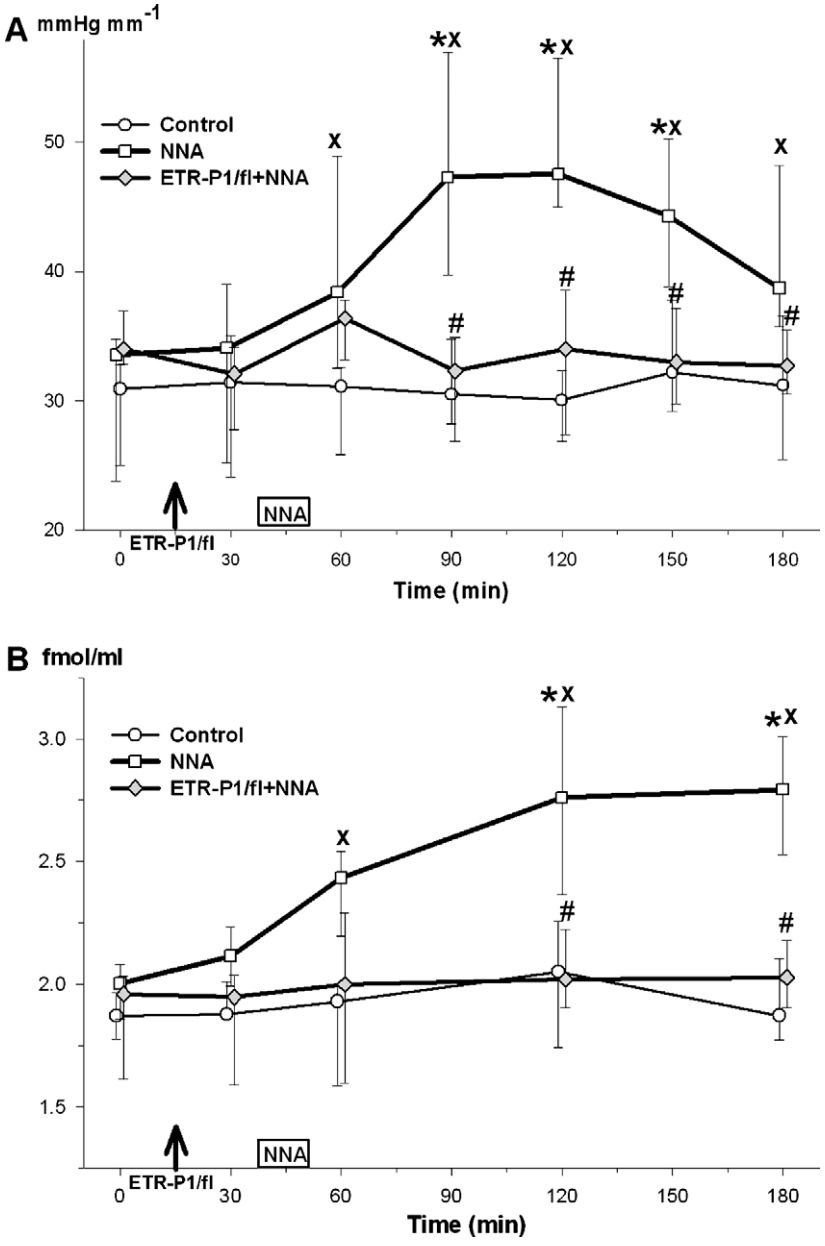

Fig. 2. Changes in myocardial contractility (A) and plasma ET-1 (B) level in the control group (black circles), the NNA-treated group (open squares) and the ETR-P1/fl + NNA-treated group (gray diamonds). $^{*}P < 0.05$ within group; $^{X}P < 0.05$ between groups *vs* saline-treated control group values; $^{\#}P < 0.05$ between groups *vs* NNA-treated group values.

Table 1
Cardiac effects of saline, NNA, endothelin-A receptor antagonist ETR-P1/fl peptide + NNA treatments on heart rate (HR) [beats/min]) and the difference between left ventricular diastolic and systolic diameters (in % baseline).

	0 min	30 min	60 min	120 min	180 min
HR					
Control	161 (149; 172)	161 (143; 180)	162 (137; 184)	156 (143; 185)	168 (149; 188)
NNA	159 (151; 169)	156 (146; 168)	126 (110; 136)[*,†]	118 (96; 125)[*,†]	120 (93; 131)[*,†]
ETR-P1/fl + NNA	159 (142; 172)	159 (147; 175)	148 (133; 164)	138 (118; 159)	146 (133; 150)
Difference between left ventricular diastolic and systolic diameters (%)					
Control	0	1.85 (0; 2.5)	−1.68 (−5.19; 0.91)	0.87 (−3.51; 2.17)	−3.47 (−9.5; 0.52)
NNA	0	−0.89 (−2.73; 0)	−12.3 (−17.3; −8.1)[†]	−19.6 (−21.6; −12.8)[*,†]	−12.7 (−22.5; −10.1)
ETR-P1/fl + NNA	0	−0.03 (−5.6; 0.51)	−2.62 (−8.5; 1.13)[‡]	−6.47 (−8.9; 2.86)[‡]	2.36 (−6.2; 4.5)[‡]

Values are medians (25th and 75th percentile).
[*] $P < 0.05$ within group.
[†] $P < 0.05$ between groups *vs* control group.
[‡] $P < 0.05$ between groups *vs* NNA-treated group.

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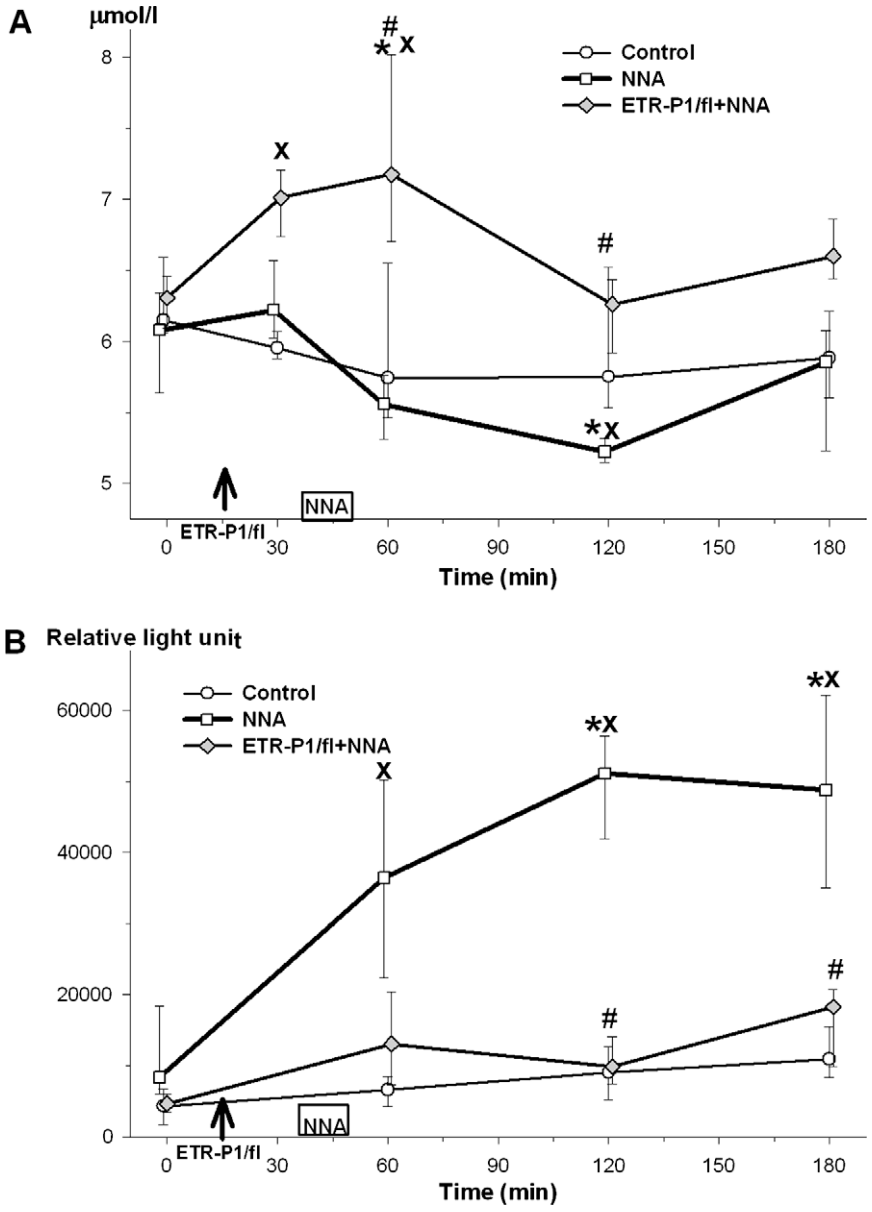

Fig. 3. Changes in plasma NO_x level (A) and superoxide production of whole blood (B) in the control group (black circles), the NNA-treated group (open squares) and the ETR-P1/fl + NNA-treated group (gray diamonds). $^{*}P < 0.05$ within group; $^{X}P < 0.05$ between groups *vs* saline-treated control group values; $^{\#}P < 0.05$ between groups *vs* NNA-treated group values.

Ca^{2+} [15]. 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+} [2,11].

Experiments with NO donors have often shown the opposing effects of NO on the myocardial contraction and heart rate. 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 heart rate, whereas higher concentrations (>100 mM) elicit negative inotropic and chronotropic effects [1,2,36].

Recent evidence has clearly demonstrated the critical role of NOS isoforms in the regulation of cardiac contractility: in NOS1-null mice, the inotropic response was suppressed, whereas in NOS3-deficient mice, the contractility was enhanced [9]. Moreover, there is a spatial confinement of NO signaling in the heart. Specifically, in the sarcoplasmic reticulum, NOS1 colocalizes with the ryanodine receptor, and the activation of NOS1 positively modulates the cardiac contractility via the augmentation of Ca^{2+} release in response to frequency and β-adrenergic receptor stimulation. In contrast, the NOS3 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 [2,9].

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 [2].

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

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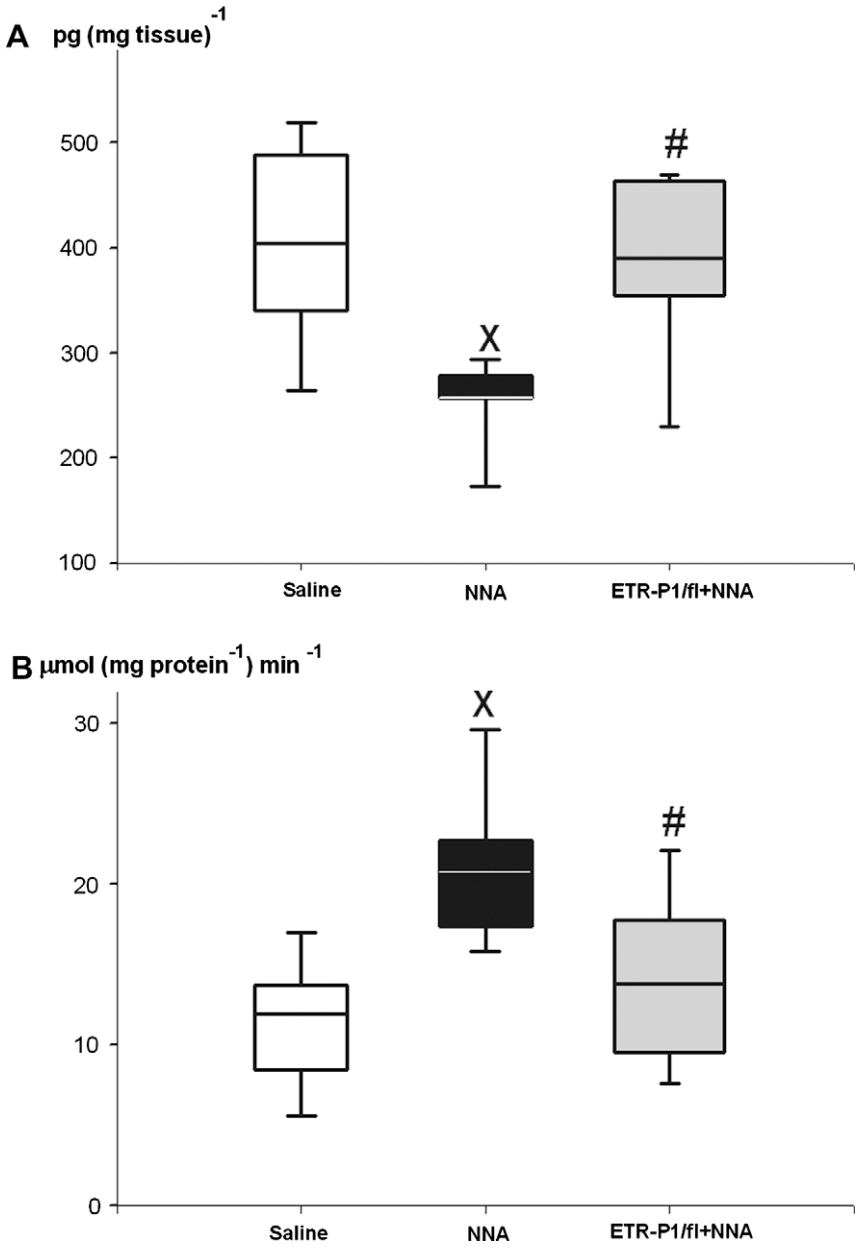

Fig. 4. Changes in ET-1 content (A) and xanthine oxidoreductase activity (B) in myocardial tissue 120 min after treatment from saline-treated control (empty box), NNA-treated (black box) and ETR-P1/fl peptide + NNA-treated (gray box) animals. The plots demonstrate the median values and the 25th (lower 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.

effectively) abrogates the modulator effect of the predominant NOS isoform.

ET-1 release and ET-1 effects on cardiac contractility

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 [18,19], the inhibition of NO synthesis can result in increased plasma levels of ET-1 [21,22]. 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 α-1, angiotensin 1 or vasopressin V1/V2 receptor activation [37].

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 [38]. Exogenous ET-1 infusion may increase the *in vivo* cardiac contractility significantly, an effect that can be prevented by ET-A receptor antagonist pretreatment [39]. 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.

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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 [2,40].

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, CI displayed an immediate significant increase and did not decline below the control level following NNA treatment, while the NOS inhibition-induced elevation in total peripheral vascular resistance was lowered. The 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 [41]. This elevation in NO production, presumably through activated ET-B receptor-induced NO release [42], has a role in normalizing the left ventricular 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.

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, and it is thereby able to decrease the plasma level of released ET-1 [33,34]. It has been shown that ETR-P1/fl peptide infusion in the same dose induces significant increase in cardiac index, left ventricular systolic–diastolic difference and significant decrease in total peripheral resistance and plasma endothelin level, while does not influence mean arterial pressure, heart rate and cardiac contractility [43].

Changes in myocardial XOR activity

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 (ROIs) in the heart, is increased in the left ventricular myocardium in nNOS-deficient mice [27,28]. Kinugawa et al. demonstrated spatially confined interactions between NOS1 and XOR [28]. 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 inhibiting effect on XOR activity *in vitro* [44]. 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 [27,44]. A deficiency of NOS1 (but not endothelial NOS) 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 [27].

Indeed, many studies have shown that superoxide radical produced by XOR decreases 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 ROIs [24,45]. Furthermore, ET-1 significantly increases intracellular superoxide levels in isolated normal cardiac myocytes [46]. 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 [46]. 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.

Limitations of the study

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 left ventricular 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 a 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.

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Conflict of interest

None declared.

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M.C. and J.K. contributed equally to this work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.niox.2009.08.003.

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