Positive effects of nitric oxide on left ventricular function in humans

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Aims The myocardial effect of tonically released nitric oxide (NO) in humans is still not known. We tested the hypothesis that low-dose NO exerts positive effects on left ventricular (LV) function.

Methods and results Twelve healthy volunteers, 26 ± 4 years, were enrolled in this study. Magnetic resonance imaging was used to precisely measure the direct effects of NO on stroke volume index (SVI). The NO pool was monitored by chemiluminescence. We reduced endogenous NO levels with intravenous infusion of the NO synthase-inhibitor NG-monomethyl-L-arginine. Replenishment of the NO pool was achieved with the NO donor S-nitrosoglutathione (GSNO) (0.5 μmol iv). To differentiate load-dependent from the direct effects of NO on LV function, changes in SVI in response to GSNO were compared with changes in the NO-independent vasodilator dihydralazine (2.5 mg iv) at matched arterial pressure and heart rate. Inhibition of NO synthesis was followed by reduction in SVI. Subsequent replenishment of the circulating NO with GSNO significantly increased SVI (39 ± 8 to 54 ± 7 mL m⁻²; P = 0.001), whereas no significant changes were observed with the NO-independent vasodilator dihydralazine (39 ± 8 to 46 ± 8 mL m⁻²; P = 0.062).

Conclusion Inhibition of endogenous NO release reduces, whereas replenishment with exogenous NO increases LV function, pointing towards a positive effect of tonically released NO on LV function in healthy humans.

Introduction

The continuous production and release of endothelial nitric oxide (NO) play an important role in vascular homeostasis. In contrast to its well-defined vasodilator effect, the role of NO for myocardial function is still a subject of significant controversy, and experimental studies suggest both positive and negative inotropic effects. Thus, the fundamental question as to whether NO contributes to the maintenance of myocardial function in humans still remains unanswered.

Initial in vitro experiments in isolated ventricular myocytes as well as in the isolated guinea pig heart suggested a negative inotropic effect of NO.1-3 A bimodal effect of NO,4 with a positive inotropic effect at low concentrations but a negative one at higher concentrations, was postulated. We recently demonstrated positive effects of tonically released NO on left ventricular (LV) function in swine in vivo.5 In humans, the results are limited and partly divergent.6-8 The direct impact of NO levels on LV function has never been determined in humans so far. Applying magnetic resonance imaging (MRI) technology, we here for the first time use a diagnostic technique for precise measurements of ventricular volumes and function9,10 to examine the effects of NO in healthy subjects. To differentiate load dependence from the direct effects of NO on LV function, changes in stroke volume index (SVI) in response to the NO-donor S-nitrosogluthathione (GSNO) were compared with the changes of the NO-independent vasodilator dihydralazine at matched arterial pressure and heart rate (HR) after the inhibition of endogenous NO synthesis. Using a refined biochemical approach, we simultaneously monitored the reduction and replenishment of the circulating NO pool. Inhibiting the endogenous NO production with the subsequent replenishment of circulating NO, we give unequivocal evidence that NO modifies baseline contractile function and that tonic release of NO constitutively sustains LV function in healthy humans.

Methods

Chemicals and solution

NG-monomethyl-L-arginine (L-NMMA) and GSNO were acquired from Clinalfa Calbiochem (Schwalbach, GER) and dissolved in 0.9% sodium
chloride. Dihydralazine (Nepresol®) was purchased from Teofarma (Pavia, Italy).

### Study population

The study population was recruited in two sets. The initial study population consisted of six healthy, physically active subjects without cardiovascular disease and was recruited among hospital staff and medical students. The preliminary results from this pilot study were used to perform a sample size calculation to determine the appropriated number of study subjects. After the first pilot experiments, additional six volunteers were recruited. All volunteers (six males, 26 ± 4 years old, 1.78 ± 0.09 m, and 70 ± 14 kg) were screened by clinical history, physical examination, and routine chemical analysis (Table 1). None of the subjects was on a regular medication, was a smoker, or revealed present or past evidence of cardiovascular diseases known to affect endothelial function, such as hypertension, hypercholesterolaemia, chronic heart failure, or diabetes mellitus. Participants in the study abstained from drinking caffeine-containing beverages for at least 12 h prior to investigation and were studied in supine position in a quiet, air-conditioned room (21 °C). There were no dropouts and all subjects completed the whole study. All subjects met the inclusion criteria. The study protocol was approved by the Ethics Committee of the local universities, and all subjects gave written informed consent before participating in the study.

### Determination of RXNO, nitrite, and nitrate

Blood was drawn from the antecubital vein, collected into a pre-chilled, heparinized tube, and centrifuged immediately for 10 min at 800g and 4 °C. Samples were stored on ice in the dark before measurement. Plasma levels of nitrolylated NO-species (RXNO: the sum of S-nitrosothiols, N-nitrosamines, iron-nitrosyl species) and nitrite were determined using a triiodide/ozone-based chemiluminescence assay, essentially as described.11,12,14 Nitrate was quantified after enzymatic reduction to nitrite by nitrate reductase using the Griess reaction as described in detail elsewhere.15

### Analysis of catecholamines

Plasma noradrenaline was determined via HPLC analysis, essentially as described.16

### Cardiac magnetic resonance imaging

Cardiac MRI was performed on a 1.0 T superconducting magnet (Gyrosan T10-NT, Intera Release 8.0, Philips) using a five-element cardiac synergy coil. Initially, a localizer sequence was acquired in the coronal, axial, and sagittal views. A vector ECG was used for ECG triggering. Fast white-blood gradient-echo (balanced fast-field echo) sequences were acquired in the breath-hold technique in the short-axis view (TR: 4.3 ms, TE: 2.1 ms; flip angle: 60 °). Depending on the size of the heart, 9—12 perpendicular short-axis slices were acquired with a slice thickness of 8.0 mm (slice gap, 0.8 mm) covering the entire ventricular volume from the apex to the aortic ventricular level. The short-axis views were transferred into a cardiac analysis program (Cardiac Application Package, Intera Release 8.0, Philips) on the local scanner console of the magnet. The bright-blood sequence provides a high contrast between myocardium and blood, with a sharp delineation of endocardial contours.17,18 The endocardial contours of the left ventricle were drawn manually with the mouse by an experienced cardio-radiologist on each slice containing 24 phase images throughout the whole cardiac cycle. The cardio-radiologist analysed the MR data in a blinded fashion. Depending on the number of short-axis views being acquired, a total number of 216—288 images were evaluated for each data set. The papillary muscles were not included into the LV volume. The software sums up the volumes of each contiguous slice in terms of a 3D determination and calculates automatically the 3D end-diastolic and end-systolic LV volumes in millilitre (mL), the stroke volume (SV) in mL, the volume-ejection fraction in percent (%), and the cardiac output (CO).19 Cardiac index (CI) and SVI were determined by adjusting for body surface area (BSA)20 such as CI = CO/BSA and SVI = SV/BSA. Repeated measurements of SVI in 12 subjects on two consecutive days showed an intra-individual variation of 5 ± 5%. Therefore, changes in SVI >10% (twice the standard deviation) were considered as relevant. Heart rate (HR) was determined from an ECG signal and calculated from the RR interval. Blood pressure was determined using a Dinamap Pro 100 monitoring system. Mean arterial pressure (MAP) was calculated as MAP = diastolic blood pressure + 1/3(systolic blood pressure — diastolic blood pressure).

### Ensuring complete NOS inhibition

To ensure the optimal l-NMMA dose for the complete inhibition of endothelial NO synthase (NOS), a dose-finding study was conducted. NO-mediatal dilation (FMD) was determined on a separate day before and after the infusion of l-NMMA as previously described.21,22 Briefly, the diameter of the brachial artery was measured using a 15 MHz transducer (Sonos 5500, Agilent) and automatic edge-detection software (Brachial Analyzer, Medical Imaging Applications, Iowa City, IO, USA), yielding a coefficient of variation of <1%. Reactive hyperaemia was induced by 5 min of distal lower arm occlusion. After 60 s, the diameter was assessed and FMD calculated as relative diameter gain compared to baseline.

### Study protocol

The study consisted of two protocols (A and B). One researcher (T.R.) was informed about the complete trial. He prepared and applied the solutions. Drugs were injected using blackened syringes with the same method of administration. The investigators analysing MRI scans and blood samples, as well as the study subjects, were blinded to treatment allocation. GSNO and dihydralazine were given in an alternating order. All subjects rested for at least 20 min to establish a stable baseline before data collection. Haemodynamic measurements were made and venous blood was collected before l-NMMA was administered intravenously via the right antecubital vein at a dosage of 1 mg kg⁻¹ min⁻¹ for 3 min followed by a continuous infusion of 0.2 mg kg⁻¹ min⁻¹ until the end of the experiment. This dosage ensured that the pharmacological inhibition of NO synthesis was as complete as possible.23 Haemodynamic measurements were repeated and the venous blood was collected after 60 min of l-NMMA infusion. At minute 64, in protocol A, a bolus of GSNO (0.5 μmol) was applied via the right antecubital vein under the continuous infusion of l-NMMA, and haemodynamic as well as biochemical measurements were repeated. The dosage of GSNO was chosen in order to replenish the reduced NO pool, and the MAP at the beginning of the experiments served as the target value. To compare the

### Table 1 Characteristics of study group

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<th>Characteristic</th>
<th>Study group 1</th>
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<td>Age (years)</td>
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haemodynamic and biochemical effects of exogenous NO with those of another potent vasodilator, all volunteers received the vasodilator dihydralazine (2.5 mg) instead of GSNO in protocol B. No adverse effects were observed in any patient during infusion of either L-NMMA or application of GSNO and dihydralazine.

**Statistical analysis**

Results are expressed as mean ± standard deviation. Data were analysed in an analysis of variance (ANOVA) with one within-subject factor (baseline vs. post-L-NMMA vs. post-GSNO or dihydralazine, respectively) and one between-subject factor (GSNO vs. dihydralazine group). A Bonferroni adjustment of the significance level was used to control the type I error for multiple pairwise comparison. Statistical significance was assumed if a null hypothesis could be rejected at $P < 0.05$ (two-sided).

Sample size calculations were performed using the G*POWER software. The sample size calculation was based on the measurement of SVI (initial $n = 6$ subjects) at baseline and after L-NMMA infusion (baseline values: 48.8 mL m$^{-2}$; post-L-NMMA: 39.8 mL m$^{-2}$). With the effect size of 1.21, $\alpha$-levels of 0.05, and a statistical power of 0.8, a sample size of $n = 12$ was calculated.

**Results**

**Inhibition of endogenous NO synthesis and haemodynamic parameters**

Consecutive to the infusion of L-NMMA, MAP was significantly increased in both groups ($P < 0.001$). This was accompanied by a significant decline in CI ($P < 0.001$), SVI ($P < 0.001$), and HR ($P = 0.001$). There were no significant differences in MAP ($P = 0.887$), CI ($P = 0.653$), SVI ($P = 0.961$), and HR ($P = 0.476$) after L-NMMA before the application of GSNO (protocol A) or dihydralazine (protocol B) (Table 2).

**Plasma NO pool**

In preliminary experiments, no significant differences in plasma nitrite after lying in supine position for 1 h ($124 \pm 21$ vs. $120 \pm 45$ nmol/L, $P = 0.645$; $n = 4$) and for 2 h ($148 \pm 49$ vs. $144 \pm 39$, $P = 0.654$; $n = 4$), respectively, without performing any intervention were seen.

When compared with baseline levels, infusion of L-NMMA was accompanied by a significant decrease in plasma nitrite levels from $159 \pm 85$ to $116 \pm 68$ nmol/L ($P < 0.001$) (protocol A) and from $151 \pm 73$ to $97 \pm 51$ nmol/L ($P < 0.001$) (protocol B), confirming the inhibition of endogenous NO production. At baseline ($P = 0.668$) and after L-NMMA ($P = 0.853$), nitrite levels between the groups (protocol A vs. protocol B) did not differ significantly. Plasma RXNOS (protocol A: $9 \pm 7$ to $7 \pm 5$ nmol/L, $P = 0.011$; protocol B: $8 \pm 5$ to $7 \pm 4$ nmol/L, $P = 0.625$) and nitrate (protocol A: $41 \pm 48$ to $37 \pm 41$ μmol/L, $P = 0.961$; protocol B: $43 \pm 41$ to $36 \pm 38$ μmol/L, $P = 0.893$) did not change from baseline levels following L-NMMA. No between-group differences were seen for RXNOS and nitrate at baseline and after infusions of L-NMMA. In addition to biochemical analyses, we measured endothelium-dependent dilation to ensure complete NOS inhibition. After 1 h of L-NMMA infusion, FMD was completely abolished ($5.64 \pm 2.52$% diameter change before to $-0.45 \pm 1.42$% following L-NMMA; $P < 0.001$; $n = 6$) in all subjects, confirming complete NOS inhibition.
When compared with dihydralazine, the application of GSNO after L-NMMA was followed by a significant increase in RXNOs (dihydralazine vs. GSNO, 4 ± 4 vs. 55 ± 12 nmol/L, \( P = 0.0005 \)) and nitrite (dihydralazine vs. GSNO, 85 ± 53 vs. 204 ± 97 nmol/L, \( P = 0.0002 \)), without affecting nitrate (35 ± 40 \( \mu \)mol/L vs. 33 ± 36 \( \mu \)mol/L; \( P = 0.068 \), dihydralazine vs. GSNO) and confirming earlier results.15

Effects of GSNO and dihydralazine on LV function

GSNO was applied under continuous L-NMMA infusion in order to replenish the reduced NO pool. Expected changes in CO and SV in response to GSNO may have occurred as a consequence of a decrease in afterload, from the activation of the sympathetic nervous system activity as a baroreflex response to the decrease in systemic blood pressure or as a direct effect of GSNO on LV function. To distinguish among these possibilities, the vasodilator dihydralazine was applied to achieve a graded decrease in systemic blood pressure, resulting in levels similar to those observed with GSNO (Table 2). At comparable levels of blood pressure and HR, the application of GSNO was followed by a significant increase in CI and SVI (Figure 1) in all subjects. Measurements of plasma noradrenaline revealed no significant differences after the application of GSNO (321 ± 96 to 375 ± 77 pg/mL) or dihydralazine (367 ± 92 to 389 ± 85 pg/mL). The increase in SVI correlated with increasing plasma RXNO concentration (Figure 2), whereas dihydralazine had no effects (Figure 3).

Discussion

The major finding of this study is that the tonic release of NO sustains LV function in healthy humans. Inhibition of NO synthesis reduced SV, whereas replenishment of the NO pool increased LV function.

Effects on LV function

Several mechanisms of positive inotropic effects of low-dose NO have been elucidated in experimental studies. These include the cGMP-mediated inhibition of phosphodiesterase and subsequently increased cAMP,24,25 a direct activation of adenyl cyclases, and26 and enhanced excitation–contraction coupling by S-nitros(yl)ation27 or by increasing contractile calcium responsiveness.3 Systemic inhibition of NO production was associated with a decline in SVI. Possible explanations include a baroreceptor reflex response to an increase in arterial pressure, a depression of the SVI due to increased afterload, and a basal myocardial NO requirement for normal contractile function. Control experiments with the \( \alpha \)-1 agonist phenylephrine to increase the arterial pressure to a level comparable with that of L-NMMA, however, showed a lesser decrease in SVI than that with L-NMMA,7 suggesting a direct myocardial action of NO. The effect of replenishment of a reduced NO pool on myocardial function in healthy humans has not been examined, yet. We applied the S-nitrosothiol GSNO on the top of the inhibited NOS activity, and the MAP at the beginning of the experiments served as the target value. This led to an increase in SVI, accompanied by a decrease in arterial blood pressure when compared with L-NMMA. To investigate whether the decrease in afterload and/or a sympathetic activation is responsible for the increase in SVI, we applied the vasodilator dihydralazine to decrease the arterial pressure to a level comparable to that resulting from GSNO. We observed no significant change in SVI after adding dihydralazine, suggesting that the effect of GSNO on cardiac function cannot be explained solely by the decrease in the arterial blood pressure. The lack of a significant difference in plasma noradrenaline after application of GSNO or hydralazine rules out a major sympathetic influence of either substance on SVI. Therefore, NO (at low concentration) improves LV function even after matching for afterload and sympathetic tone pointing towards a direct NO-related effect on the myocardium.

NO levels and LV function

The source of NO that exerts positive effects on LV function is unclear. It may be of myocardial as well as of systemic origin. Potential myocardial sources can be enzymatic or non-enzymatic. Besides the myocardial source, a circulating NO pool exists. NO circulates in plasma as nitrite, nitrate and as nitros(yl)ated species (RXNO: the sum of S-nitrosothiols, N-nitrosamines, iron-nitrosyl species). Utilizing a pharmacological approach, here we reduced the NO levels by infusion of the NOS-inhibitor L-NMMA. GSNO

![Figure 1](https://example.com/figure1.png) Changes in SVI after the application of L-NMMA (referring to basal; protocol B), dihydralazine (referring to L-NMMA; protocol B), L-NMMA (referring to basal; protocol A), and GSNO (referring to L-NMMA; protocol A) \( (n = 12) \).
Figure 2  SVI in relation to the plasma RXNO concentration in 12 subjects. Protocol A: (A) Levels after the application of L-NMMA in relation to baseline levels. (B) The consecutive application of GSNO led to an increase in the plasma RXNO that was paralleled by an increase in SVI.

Figure 3  SVI in relation to the plasma RXNO concentration in 12 subjects. Protocol B: (A) Levels after the application of L-NMMA in relation to baseline levels. (B) Levels after L-NMMA compared with dihydralazine.
was then given in a nanomolar concentration to replenish the NO pool. Applying a refined biochemical approach, we provide here a sensitive analytical method to monitor the reduction as well as the replenishment of the NO pool. Infusions of L-NMMA were associated with a decrease in plasma nitrite and RXNOS. The only slight reduction of RXNOS after L-NMMA may have two reasons: the long half-life of these species or the still incomplete inhibition of the NO synthesis. However, the complete inhibition of NO could be shown by the abolishment of FMD after L-NMMA. GSNO led to an increase in plasma levels of RXNO and nitrite. The absolute NO levels necessary for maintaining LV function are unknown. However, no matter how the absolute levels of NO are and where the NO is derived from, the replenishment of the reduced circulating NO pool results in a consistent increase in SV in all subjects (Figure 2 compared with Figure 3 after the application of dihydralazine).

Conclusions

Our data clearly demonstrate that the tonic release of NO exerts positive effects on LV function in humans in vivo. However, the sample size in our study is relatively small. We observed changes in SVI after dihydralazine. It would require larger studies to confidently conclude that the change in SV after adding S-nitrosoglutathione was significantly larger than that after dihydralazine. Recently, it has been shown that isosorbide dinitrate with hydralazine in addition to the standard therapy provides additional benefit in blacks with advanced heart failure. In the present study, we only examined healthy subjects. Whether these results obtained here in healthy subjects can be extrapolated to patients with congestive heart failure deserves further studies. Here, we focused on baseline NO levels in maintaining LV function, with NO being mainly derived from constitutively expressed eNOS. Future studies will have to show whether the replenishment of reduced NO levels may be crucial for maintaining LV function in, for example, myocardial infarction. In conditions with cytokine-induced increase of inducible NOS expression and activity, such as severe sepsis, the formation of high amounts of NO depresses myocardial function. Thus, because of the bimodal effects of NO, with a positive effect on LV function at low amounts and a negative one at high amounts, the threshold level for the switch from a positive to a negative effect of NO seems to be of importance.

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References


