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THE EFFECTS OF MODULATION OF ENDOTHELIAL DERIVED NITRIC OXIDE LEVEL ON CONTRACTILE FUNCTION AND MYOCARDIAL OXYGEN CONSUMPTION IN SALINE PERFUSED MOUSE HEART

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*The aim of the study was to estimate myocardial oxygen consumption during modulation of nitric oxide levels by stimulating or inhibiting endogenous nitric oxide synthesis and by applying nitric oxide exogenously. Isolated mouse hearts (n=97) were paced and perfused at constant flow (16.0 ± 0.3 ml*g^{-1}*min^{-1}). Basal contractility and myocardial oxygen consumption were 2.30 ± 0.02 mmHg*ms^{-1} and 10.7 ± 0.2 μmol*g^{-1}*min^{-1}, respectively. Maximal stimulation of nitric oxide formation by bradykinin (10 μmol*l^{-1}) increased coronary venous nitrite release 5-fold to 960 pmol*g^{-1}*min^{-1} (n=17). Vasodilatation, induced by either bradykinin or adenosine caused a fall in perfusion pressure and a distinct decrease in contractility and myocardial oxygen consumption. Therefore, all further experiments were conducted at the maximal vasodilatation (adenosine, 1 μmol*l^{-1}). Under these conditions, no effect of bradykinin on contractility and oxygen consumption rate was seen. Also, authentic nitric oxide in concentrations less than 2 μmol*l^{-1} did not alter either contractility or myocardial oxygen consumption. Only concentrations of nitric oxide higher than 5 μmol*l^{-1} induced contractile dysfunction and reduction in myocardial oxygen consumption. Finally, inhibition of nitric oxide synthase had only marginal effects (n=6). Thus in the saline perfused mouse heart, neither the basal release of nitric oxide nor the bradykinin induced stimulation of endothelial nitric oxide formation are quantitatively sufficient to impair myocardial oxygen consumption.*

Key words: contractility, heart, nitric oxide, oxygen consumption.

INTRODUCTION

Even before nitric oxide (NO) was discovered to be the responsible agent, it was known that activated macrophages produced a substance that was cytotoxic to other cells by irreversibly inhibiting their mitochondrial respiration (Granger, 1980). In 1994 a radically different effect of NO on mitochondrial respiration was reported (Brown and Cooper, 1994; Cleeter *et al.*,1994). Very low (nanomolar) levels of NO caused a completely reversible inhibition of mitochondrial respiration at cytochrome oxidase in competition with oxygen. This raised the exciting

possibility that NO is a physiological regulator of mitochondrial respiration (Brown, 1995). Potential targets involve inhibition of mitochondrial oxidative phosphorylation at the level of cytochrome *c* oxidase and inhibition of mitochondrial or cytosolic creatine kinase. NO may also govern substrate utilization. Moreover, NO may influence contractility and thus energy demand by cGMP mediated modulation of L--type Ca^{2+}-channel activity and cGMP mediated phosphorylation of phospholamban, enhancing SR Ca^{2+}-ATPase activity (Brown 1995).

While the mitochondrial respiratory chain is very sensitive to blockade by nitric oxide (Koivisto *et al.*, 1997), it is still unclear whether the basal concentration of nitric oxide in the mitochondria vicinity is quantitatively sufficient to exert a tonic control of oxidative phosphorylation and oxygen consumption. In fact, some studies (Bernstein *et al.*, 1996; Decking *et al.*, 1995) showed that blockade of nitric oxide synthase increased myocardial oxygen consumption(<12%), but others did not (Duncker *et al.*, 2000; Xie *et al.*, 1996), enhancing endothelial nitric oxide formation in the heart *in situ* also led to conflicting results (Mital *et al.*, 2000; Crystal and Gurevicius, 1996). Only when studying isolated myocardial tissue pieces, did elevated endothelial nitric oxide formation consistently decrease myocardial oxygen consumption (Xie *et al.*, 1996; Loke *et al.*, 1999). These data have been taken to show that endothelial nitric oxide formation exerts a direct effect on the oxygen consumption of the surrounding cardiomyocytes. In order to test this hypothesis, the effects of bradykinin-enhanced nitric oxide formation on myocardial oxygen consumption rate and contractile function were studied in the saline perfused mouse heart. Myocardial nitric oxide levels were also modulated by applying authentic nitric oxide or an inhibiting cardiac nitric oxide synthesis.

MATERIALS AND METHODS

General

A total of 97 C57/BL6 mice, of body mass between 20 and 30 grams, were anesthetized by intraperitoneal injection of urethane (1.5 mg/kg). Heparin was given simultaneously. Hearts with a wet weight of 116 ± 11 mg were rapidly excised, the aorta was cannulated (20-gauge stainless steel, inner diameter 0.6 mm, outer diameter 0.9 mm) and hearts were perfused at a pressure of 100 mmHg in a non-recirculating Langendorff mode with modified Krebs-Henseleit buffer. It contained (in mM): NaCl 116, KCl 4.6, $MgSO_4$ 1.1, $NaHCO_3$ 24.9, $CaCl_2$ 2.5, KH_2PO_4 1.2, glucose 8.3, pyruvate 2.0 and EDTA 0.5 and was equilibrated with 95% O_2 and 5% CO_2 (pH 7.4, 37 ^oC). The perfusion system (Isolated Heart size 1, HSE Harvard Apparatus, Germany) enabled the temperature control of both inflow medium and ambient air. The investigation conformed 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).

Small silver electrodes were gently applied to the right atrium and cardiac apex for cardiac pacing. The following parameters were continuously recorded (Powerlab, ADInstruments, Castle Hill, Australia): coronary flow as measured by a

transit-time ultrasonic flowmeter (HSE Harvard Apparatus, Germany), coronary perfusion pressure (CPP), left intraventricular pressure (LVP) as assessed by a fluid-filled balloon, heart rate, and coronary venous pO_2. For reliable pO_2 measurements, a fraction of the coronary venous effluent (0.5 ml*min^{-1}) was sucked continuously through a small diameter tube placed in the opening of the pulmonary artery across a Clark type pO_2 electrode (733, Diamond General, USA) by a peristaltic pump (Minipulse 3, Gilson Medical Electronics, France). For the measurement of nitrite release, coronary venous effluent was collected as well.

Protocols

After completion of the preparation, hearts were initially perfused at constant pressure (100 mmHg) inside a water-jacketed chamber set to 37 ^oC. Cardiac pacing (500 min^{-1}) was initiated and continued throughout. All hearts were allowed to equilibrate for 20 minutes before left ventricular end diastolic pressure was set to 5 mmHg.

Twenty minutes after the onset of cardiac pacing, the coronary perfusion rate was fixed to the steady state flow finally attained (in general about 1.5 – 2.5 ml*min^{-1}) and was maintained constant thereafter by a peristaltic pump. Basal functional parameters were acquired before subjecting the hearts to the different experimental protocols. In the first experiments, bradykinin only was applied (n=17). Since under conditions of constant coronary flow, bradykinin induced a massive decrease in perfusion pressure affecting contractility to a significant extent, in the main set of experiments (n=80), maximal vasodilatation was initiated by application of adenosine (1 μmol*l^{-1}) which was continued throughout, before starting the different interventions under steady-state conditions. Bradykinin (10 μmol*l^{-1}), N^G-monomethyl-L-arginine (100 μmol*l^{-1}) or authentic nitric oxide solution (33 nmol*l^{-1} – 20 μmol*l^{-1}) was applied in the presence of 1 μmol*l^{-1} adenosine. At the end of the experiment, hearts were weighed after gentle blotting.

Measurement of myocardial oxygen consumption

To enable the sensitive detection of even small changes in myocardial oxygen consumption, hearts were perfused at a constant arterial pO_2 (600 mmHg) and flow. In brief, measurement of the coronary venous pO_2 (see above) allowed the determination of the arterio-venous pO_2 difference. Since flow was maintained constant, any change in oxygen consumption translated into a considerable change in coronary venous pO_2. Preliminary experiments demonstrated both the stability of the pO_2 electrode's signal at a given pO_2 (electrode drift less than 0.1 mmHg *min^{-1}) as well as the ability of the set-up to respond to changes in myocardial oxygen consumption.

Nitrite release measurements

To obtain a measure of cardiac nitric oxide formation, the nitrite release of the saline perfused hearts was determined. Measurement of nitrate release was not attempted, since it is complicated by a low nitrate content of the arterial inflow

medium due to contamination of the commonly available medium constituents. The arterial inflow and coronary venous outflow nitrite concentration was measured by a nitric oxide analyser (NOA, Model 280, Sievers, Boulder, USA) based on the chemiluminescence method. Comparison with nitrite standards enabled quantification, the detection limit being 1 pmol. To measure reliably the low coronary nitrite concentrations, an injection volume of 500 μl was employed. For standards in the range from 1 – 200 nmol*l^{-1} nitrite, a linear correlation coefficient r of 0.98 was obtained.

Chemicals

Bradykinin, N^G-monomethyl-L-arginine, and urethane were obtained from Sigma (Deisenhofen, Germany) and heparin was purchased from Hoffmann-LaRoche (Grenzau, Germany). All other reagents were obtained from Merck (Darmstadt, Germany). Nitric oxide gas was obtained from AGA gas, Hamburg, Germany.

Data analysis

For statistical analysis, two tailed *t* tests for paired or unpaired data were used as appropriate. A p value less than 0.05 was considered significant. All data are given as means ± SEM.

RESULTS

Effect of increased endothelial nitric oxide-formation on myocardial contractile function and oxygen consumption

To increase the level of endothelium-derived nitric oxide, bradykinin (10 μmol*l^{-1}) was applied. The effects of bradykinin on cardiac functions and oxygen consumption were first analyzed in the isolated mouse heart perfused with constant a coronary flow at basal vascular tone (n=17). When bradykinin was applied, a major fall in perfusion pressure was observed in association with a decrease in contractility and myocardial oxygen consumption. This apparent decrease was due to the decrease in perfusion pressure, since adenosine (1 μmol*l^1, n = 12), an endothelium independent agent, had a quantitatively comparable effect (Table 1). It suggests that the effect noticed with bradykinin seems to be primarily caused by vasodilatation.

To avoid the effect of vasodilatation on coronary perfusion pressure, contractility and myocardial oxygen consumption, in the subsequent series of experiments the coronary blood vessels were maximally dilated by previous and continuous infusion of adenosine (1μmol*l^{-1}). As a consequence, when adenosine-induced vasodilatation with its concomitant decrease in perfusion pressure, contractility and myocardial oxygen consumption preceded application of bradykinin, bradykinin did not result in any further decrease in contractility and myocardial oxygen consumption (Table 2, Figure 1).

Table 1. Comparison of the effects of nitric oxide-dependent (bradykinin 10 μmol*l^{-1}) and nitric oxide-independent vasodilatators (adenosine 1μmol*l^{-1}) on changes in cardiac function and myocardial oxygen consumption (MVO_2) of the constant-flow perfused mouse heart.

	CPP (mmHg)	LV dP/dt_{max} (mmHg/ms)	MVO_2 (μmol*g^{-1}*min^{-1})
Basal value	100.0±0.6	2.30±0.02	10.7±0.2
Bradykinin	51.0±.0	1.80±0.02	10.1±0.5
Adenosine	42.0±4.0	1.68±0.06	9.6±1.0

Cardiac function is expressed as coronary perfusion pressure (CPP), maximal rate of change of left ventricular pressure with time (LV dP/dt_{max}) and myocardial oxygen consumption (MVO_2). Basal value: n=97, Bradykinin: n=17, Adenosine: n=12. Data expressed as mean ± SEM.

Table 2. Lack of effect of bradykinin (10 μmol*l^{-1}) on coronary perfusion pressure (CPP), maximal rate of change of left ventricular pressure with time (LV dP/dt_{max}) (contractility) and myocardial oxygen consumption (MVO_2) in the presence of maximal vasodilatation induced by adenosine (1 μmol*l^{-1}) (n=12). Coronary flow was fixed throughout the experiment.

	CPP (mmHg)	LVDP (mmHg)	LV dP/dt_{max} (mmHg/ms)	MVO_2 (μmol*g^{-1}*min^{-1})
Basal value	100±7	84±4	2.30±0.06	10.40±0.80
Adenosine	49±3	64±4	1.75±0.06	9.83±0.73
Adenosine+Bradykinin	50±3	66±5	1.80±0.06	9.79±0.68

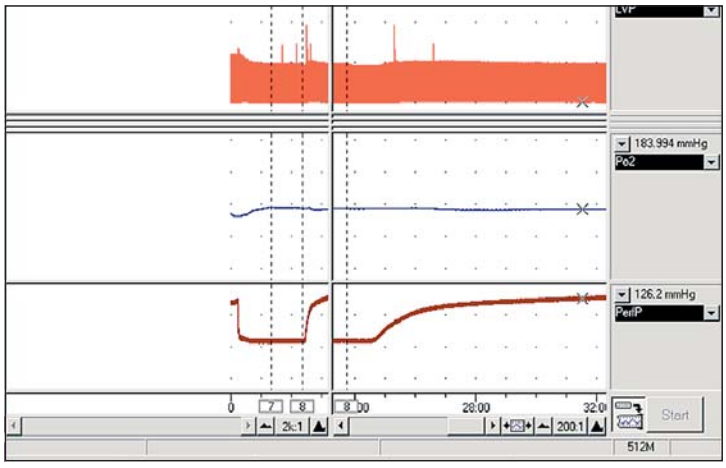

Figure 1. Original trace - Lack of effect of bradykinin (10 μmol*l^{-1}) on contractility (maximal rate of change of left ventricular pressure with time - LV dP/dt_{max}) and myocardial oxygen consumption (MVO_2) in presence of maximal vasodilatation induced by adenosine (1 μmol*l^{-1}) (n=12).

Exogenous application of nitric oxide

To gain an insight into the nitric oxide concentration necessary to depress the cardiac contractile function and myocardial oxygen consumption significantly, a concentration-response curve for exogenously applied authentic nitric oxide was obtained, again in the presence of adenosine (1 μmol*l^{-1}). As shown in trace 2 (Figure 2), in the concentration range less than 2 μmol*l^{-1}, no effect of nitric oxide on either contractile function or myocardial oxygen consumption was noticed (n=9). Only at concentrations higher than 2 μmol*l^{-1}, nitric oxide impaired the contractile function and decreased myocardial oxygen consumption in a concentration-dependent manner (n=6).

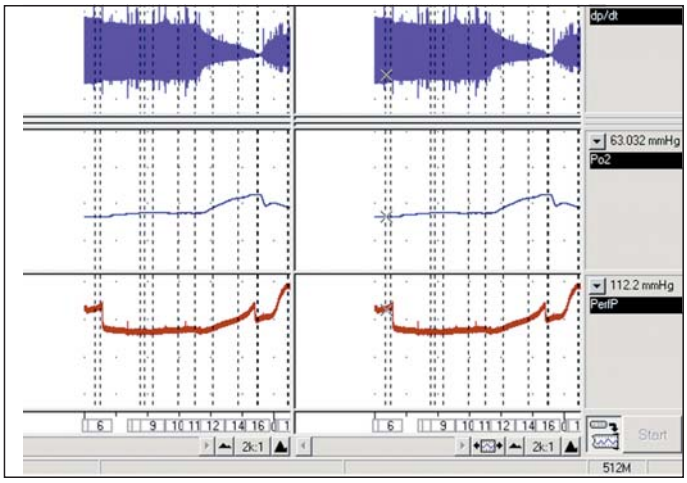

Figure 2. Original trace - Effect of arterially applied authentic nitric oxide solution (30 nmol*l^{-1} - 20 μmol*l^{-1}) on contractile function (maximal rate of change of left ventricular pressure with time LV dP/dt_{max}) and myocardial oxygen consumption (MVO_2) in the presence of adenosine (1μmo*l^{-1}) (n=6-9 for each concentration). Coronary flow was fixed thoughout the experiment.

Nitrite release

To assess the rise in nitric oxide induced by bradykinin (10 μmol*l^{-1}), exerting a maximal vasodilatory effect, coronary venous nitrite release was determined. The basal coronary venous nitrite concentration was 23 ± 3 nmol*l^{-1}. Following the correction for the arterial inflow nitrite concentration of 13 nmol*l^{-1}, basal myocardial nitrite release was determined to be 192 pmol*g^{-1}*min^{-1}. Bradykinin induced a 5-fold rise in nitrite release (to 960 pmol*g^{-1}*min^{-1}), the coronary venous effluent concentration being 66 ± 8 nmol*l^{-1} (Figure 3).

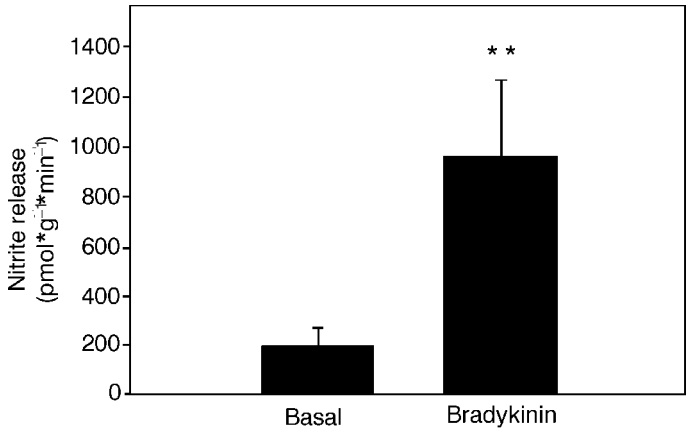

Figure 3. Nitrite release (pmol*g^{-1}*min^{-1}) in the coronary venous effluent under basal conditions (Basal) and during application of bradykinin (Bradykinin). Significance mark is ** $p<0.01$ vs.basal (n=17).

Decreased level of endogenous endothelial nitric oxide formation after inhibition of nitric oxide synthase

To test whether the basal formation of nitric oxide contributes to a tonic inhibition of myocardial oxygen consumption, endothelial nitric oxide synthesis was blocked by N^G-monomethyl-L-arginine (L –NMMA) (100 μmol*l^{-1}), again during maximal vasodilatation (adenosine,1μmol*l^{-1}). Under these conditions, L-NMMA induced a small rise in coronary perfusion pressure (46.3 ± 1.5 vs. 44.6 ± 1.4 mmHg) and contractility (1.9 ± 0.1vs.1.84 ± 0.07 mmHg*ms^{-1}), but had no effect on oxygen consumption. Thus, oxygen consumption was 9.93 ± 2.60 and 9.87 ± 2.70 %, respectively, of arterially supplied oxygen with or without nitric oxide synthase blockade by L-NMMA (n=6).

DISCUSSION

The present study demonstrates that a 5-fold rise in endothelial nitric oxide formation does not act on contractile function and myocardial oxygen consumption in the saline perfused mouse heart when the confounding effects of vasodilatation are carefully controlled. When modulating myocardial nitric oxide levels using arterial application of authentic nitric oxide, concentrations more than 2 μmol*l^{-1} of nitric oxide were required to induce a decrease in myocardial oxygen consumption. This concentration is high when compared to the basal nitrite release of about 10 nmol*l^{-1}. Consistent with these data, blockade of nitric oxide synthase by L-NMMA showed no effect on myocardial oxygen consumption. There was thus no direct evidence to support the idea of a tonic control of oxygen consumption by endogenous nitric oxide formation. In fact, very high arterial nitric oxide concentrations were needed to inhibit myocardial oxygen consumption.

Based on these observations, we conclude that endothelial-derived nitric oxide exerts no control on mitochondrial oxygen consumption, most likely due to the rapid degradation of nitric oxide, e.g. by myoglobin.

The main data of the present study appear to be in conflict with the recent observation of a substantial decrease in myocardial oxygen consumption upon bradykinin in cardiac tissue (Xie *et al.*, 1996; Loke *et al.*, 1999; Pittis *et al.*, 2000). The reason for this discrepancy could be as followed:

- The bradykinin-concentration in the present study is comparable to the concentrations used in these recent observations (Loke *et al.*, 1999; Pittis *et al.*, 2000). This concentration was more than 10-fold greater than the maximally effective vasodilatory concentration in mice (Flögel *et al.*, 2001). Thus, an insufficient bradykinin-concentration cannot explain the present lack of effect.
- To exclude insufficient nitric oxide formation in the isolated mouse heart, we determined the net nitrite release as a commonly employed index of cardiac nitric oxide formation. Basal nitrite release (10 $nmol*l^{-1}$) was similar to that of guinea pig hearts (Kelm *et al.*, 1997). The bradykinin-induced rise (4-fold) in nitrite by far exceeded that seen in canine coronary microvessels (Pittis *et al.*, 2000) and in guinea pig hearts (Kelm and Schrader, 1997). Thus, impaired nitric oxide formation seems to be unlikely.
- Adequate myocardial oxygenation is critical and indicated both by the level of free energy of ATP hydrolysis (63 kJ/mol) (Flögel *et al.*, 1999) and by a high myocardial oxygen consumption (10 $\mu mol*g^{-1}*min^{-1}$). In contrast, when myocardial tissue pieces were studied, myocardial oxygen consumption ranged from 0.07 to 0.2$\mu mol*g^{-1}*min^{-1}$ only (Loke *et al.*, 1999; Pittis *et al.*, 2000), strongly suggesting insufficient oxygenation, at least in the tissue core.

We would therefore like to suggest that the discrepancy between bradykinin's effect on myocardial oxygen consumption in isolated hearts and tissue pieces is at least partially explained by differences in pO_2. It is well known that the inhibitory action of nitric oxide at cytochrome *c* oxidase is competitive with regards to oxygen (Koivisto *et al.*, 1997). A low pO_2 will thus facilitate inhibition of oxidative phosphorylation by nitric oxide and result in a high sensitivity of tissue pieces to bradykinin-induced nitric oxide formation. However, this is not the case in the saline perfused heart.

The rate of bioactive nitric oxide formation in the heart *in vivo* is most likely similar to the saline perfused heart. Tonic nitric oxide formation contributes to the maintenance of vascular tone but it is below the threshold for maximal vasodilatation. *In vivo*, the presence of hemoglobin presents a further source for nitric oxide degradation and inactivation. In consequence, in the heart *in vivo* it is not likely that endothelial nitric oxide formation contributes to the control of mitochondrial respiration.

Against this background, it is difficult to understand, why nitric oxide synthase blockade, e.g. in guinea pigs and dogs (Bernstein *et al.*, 1996; Decking *et al.*, 1995) induced a rise in myocardial oxygen consumption. It is possible that

nitric oxide, formed within the mitochondria, is responsible for this effect. Mitochondrial nitric oxide formation would enable direct inhibition of the respiratory chain by nitric oxide and bypass the potent inactivation of nitric oxide by myoglobin recently demonstrated by us (Flögel *et al.*, 2001). It seems to be most likely that this degradation pathway made the application of very high arterial nitric oxide concentrations necessary to induce the contractile dysfunction and decline in myocardial oxygen consumption. In addition, this nitric oxide inactivation makes tonic control of mitochondrial oxidative phosphorylation by endothelium-derived nitric oxide hardly possible.

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EFEKTI MODULACIJE NIVOA ENDOTELNOG AZOT MONOKSIDA NA KONTRAKTILNU FUNKCIJU I POTROŠNJU KISEONIKA U IZOLOVANOM PERFUNDOVANOM SRCU MIŠA

KOJIC ZVEZDANA

SADRŽAJ

Cilj ovog rada bio je da se ispita kontraktilna funkcija srca i potrošnja kiseonika za vreme modulacije nivoa azot monoksida (NO) u izolovanom srcu miša koje je perfundovano fiziološkim rastvorom pri konstantnom koronarnom protoku. Modulacija nivoa NO ostvarena je putem inhibicije (L-NMMA) ili stimulacije (bradikininom) endotelne azot monoksid sintaze, kao i putem egzogene primene autentičnog rastvora NO.

U bazalnim uslovima, pri koronarnom protoku od 16.0 ± 0.3 $ml*g^{-1}*min^{-1}$, koncentracija nitrita u koronarnom venskom efluentu iznosila je 192 ± 40 $pmol*g^{-1}*min^{-1}$, potrošnja kiseonika (MVO_2) iznosila je 10.7 ± 0.2 $\mu mol*g^{-1}*min^{-1}$ a kontraktilnost srca, izražena kroz indeks dP/dt leve komore iznosila je 2.30 ± 0.02 $mmHg*ms^{-1}$. Bradikinin (10 $\mu mol*l^{-1}$) je doveo do petostrukog porasta koncentracije oslobođenih nitrita (do 960 $pmol*g^{-1}*min^{-1}$), koji je bio udružen sa velikim padom potrošnje kiseonika, kontraktilne funkcije srca i koronarnog perfuzionog pritiska (n=17). Međutim, sličan pad MVO_2 i LV dP/dt registrovan je i u toku upotrebe NO-nezavisnog vazodilatatora (adenozin, 1 $\mu mol*l^{-1}$).

Zbog toga, da bi izbegli efekat promene koronarnog vaskularnog tonusa, u svim daljim eksperimentima (n=80) maksimalna vazodilatacija je predhodno ostvarena adenosinom. U prisustvu maksimalne vazodilatacije, primena bradikinina, L-NMMA-a i niskih koncentracija autentičnog rastvora NO (manje od 2 $\mu mol*l^{-1}$) nije imala, ili je imala samo neznatni efekat na kontraktilnu funkciju i na potrošnju kiseonika u srcu miša. Samo autentični rastvor NO u koncentracijama većim od 5 $\mu mol*l^{-1}$ izazvao je kontraktilnu disfunkciju i značajno smanjenje potrošnje kiseonika u srcu.

U uslovima dobre kontrole koronarnog vaskularnog otpora samo koncentracije egzogenog rastvora NO veće od 5 $\mu mol*l^{-1}$ značajno su smanjile potrošnju kiseonika i kontraktilnu funkciju srca. Bazalne vrednosti NO kao i vrednosti NO koje nastaju u toku endogene stimulacije endotela bradikininom, mnogo su manje i kvantitativno nisu dovoljne da bi vršile značajnu toničku inhibiciju potrošnje kiseonika u miokardu.