

## Original papers

# Opioidergic conditioning of the human heart muscle in nitric oxide-dependent mechanism

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D – writing the article; E – critical revision of the article; F – final approval of the article

Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online)

*Adv Clin Exp Med.* 2018;27(8):1069–1073

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## Funding sources

None declared

## Conflict of interest

None declared

Received on February 18, 2016

Reviewed on November 28, 2016

Accepted on April 4, 2017

## Abstract

**Background.** Opioidergic conditioning is well documented to trigger cardioprotection against ischemia/reperfusion (I/R) injury. Previous studies on animal models have suggested that nitric oxide (NO) mediates the beneficial effect of opioids, but the role of NO in humans seems to be controversial.

**Objectives.** The aim of the study was to assess the influence of NO modulators on opioid-induced cardioprotection in the human myocardium.

**Material and methods.** Trabeculae of the human right atria were electrically driven in an organ bath and subjected to simulated I/R injury. The non-selective inhibitor of nitric oxide synthase (NOS) – *N*-methyl-*L*-arginine (LNMMA), the donor of NO – *S*-Nitroso-*N*-acetylpenicillamine (SNAP) or morphine (in the amount of  $10^{-4}$  M) were used at the time of re-oxygenation. The additional trabecula was subjected to the hypoxia protocol only (control). The contractility of the myocardium was assessed as the maximal force of a contraction ( $A_{max}$ ), the rate of rise of the force of a contraction (Slope L) and the cardiac muscle relaxation – as the rate of decay of the force of a contraction (Slope T).

**Results.** The application of 100  $\mu$ M LNMMA resulted in the decrease of  $A_{max}$ , Slope L and Slope T during the re-oxygenation period as compared to control. The application of  $10^{-4}$  M morphine and/or 100  $\mu$ M SNAP resulted in a partial reversal of the detrimental influence of LNMMA.

**Conclusions.** At the re-oxygenation period, the blockade of NO synthesis has a deleterious effect on the systolic and diastolic function of the human myocardium as well as attenuates the beneficial effect of morphine conditioning.

**Key words:** ischemia, nitric oxide, reperfusion, morphine

## DOI

10.17219/acem/70192

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## Introduction

Ischemic heart disease is known as the leading cause of morbidity and mortality in adults. Early reperfusion is necessary to restore perfusion of the ischemic heart muscle. However, reperfusion may induce a cascade of pathophysiological reactions, increasing the infarct area of the myocardium by up to 50% of the final size.<sup>1</sup> Sequences of brief episodes of non-lethal ischemia and reperfusion applied before (ischemic preconditioning – IPC) or after (ischemic postconditioning – POC) the coronary occlusion are well documented to reduce ischemia/reperfusion (I/R) injury. Regarding the fact that applying these techniques in humans is impractical, as well as that the results from human trials have been controversial, extensive research efforts have been made to find pharmacological agents which can mimic these cardioprotective strategies.<sup>2–4</sup> The mechanisms underlying IPC or POC are still not clarified, but strong experimental evidence suggests that opioids may be a part of the endogenous cardioprotective response to I/R injury and trigger intracellular enzyme cascades, leading ultimately to the closure of the mitochondrial permeability transition pores (mPTP), responsible for the induction of cell damage.<sup>5</sup> Previous studies on animal models have suggested the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) signaling as the main pathway involved in the beneficial effect of opioids, but our understanding of the role of nitric oxide synthases (NOS) in modulating I/R injury in humans remains limited. We hypothesize that the opioid receptor activation provides cardioprotection in the human heart muscle through a NO-dependent pathway, which may give insight into explaining the protective mechanisms against I/R injury.

## Material and methods

### Material

The experiments were performed on muscular trabeculae obtained from the right heart atrial appendages of 58 consecutive patients (35 males and 23 females) subjected to the coronary artery bypass surgery. Patients diagnosed with significant valvular heart disease or with severe heart failure were excluded from the study. The patients' demographic data is presented in Table 1.

### Methods

Fragments of the human right heart atria were transported from the cardiac surgery room to the laboratory in the ice-cold Krebs-Henseleit solution ([M]: 118.0 NaCl, 4.70 KCl, 1.52 CaCl<sub>2</sub>, 1.64 MgSO<sub>4</sub>, 24.88 NaHCO<sub>3</sub>, 1.18 KH<sub>2</sub>PO<sub>4</sub>, 11.0 glucose, and 2.0 sodium pyruvate; pH 7.4). Two muscular trabeculae were dissected from the right heart atria and incubated in 2 separate organ baths (Schuler Organ

**Table 1.** The patients' demographic data, preoperative drug treatment and preoperative left ventricular ejection fraction

1	men/women, n	35/23
2	age [years]	62.8 ± 5.7
3	ejection fraction, mean ± SD	52.3 ± 2.39%
4a	diabetes, n (%)	12 (20)
4b	diabetes with insulin treatment, n (%)	7 (12)
5	Drugs, n (%)	
5a	beta-blockers	43 (75)
5b	calcium channel blockers	10 (18)
5c	angiotensin II converting enzyme inhibitors	30 (52)
5d	angiotensin II receptor blockers	2 (3)
5e	statins	40 (69)

SD – standard deviation.

Bath, Hugo Sachs Elektronik – HSE, March-Hugstetten, Germany), both filled with the Krebs-Henseleit solution warmed up to 37°C. To avoid core hypoxia, the trabeculae included in the study had a cross-sectional area <1 mm in diameter. Two trabeculae from each patient were always studied simultaneously and exposed to the hypoxia protocol including 60 min of hypoxia (incubation in the Krebs-Henseleit buffer deprived of glucose and pyruvate saturated with 95% argon and 5% carbon dioxide) with subsequent 60 min of re-oxygenation (incubation in the Krebs-Henseleit buffer saturated with the 95% oxygen and 5% carbon dioxide). The buffer was replaced every 15 min, except the time of hypoxia. Every trabecula was stretched to 90% of its optimal tension strength according to the Frank-Starling relationship, and all trabeculae were driven throughout the experiments with 1 Hz 50 ms square stimuli, using platinum field electrodes and a stimulator (Type 215, HSE). The contractive function of every trabecula was recorded with the use of a transducer (Type 372, HSE). The signal was enhanced with a bridge amplifier (Type 336, HSE), and recorded by a PowerLab/4SP system and analyzed off-line using Chart software (ADInstruments, Chalgrove, UK). Each experimental protocol was completed with the application of 10 µM of norepinephrine (NE) to assess the viability of the trabeculae.

### Protocols

To determine the effects of modulation of the NO pathway, the non-selective inhibitor of NOS – *N*-methyl-*L*-arginine (LNMMMA), the donor of NO – *S*-nitroso-*N*-acetylpenicillamine (SNAP) or morphine (in the amount of 10<sup>-4</sup> M) were used at the time of re-oxygenation. The other trabecula was subjected only to the hypoxia protocol (control). The experimental protocols are depicted in Fig. 1.

The contractility of the myocardium assessed as the maximal force of a contraction (maximal amplitude of the peak – A<sub>max</sub>), the rate of rise of the force of a contraction (the slope of the leading edge of the peak

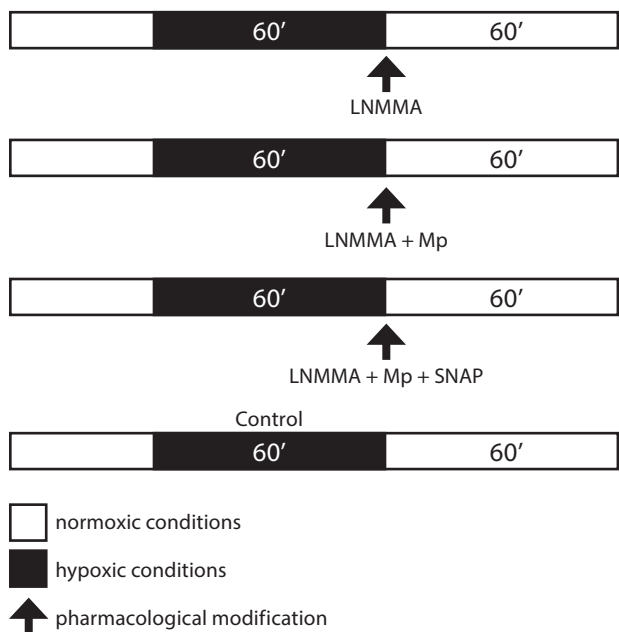


Fig. 1. Protocols for the experimental groups

Mp – morphine.

– Slope L) and the cardiac muscle relaxation – as the rate of decay of the force of a contraction (the slope of trailing edge of the peak – Slope T) were obtained in the 5<sup>th</sup>, 10<sup>th</sup>, 15<sup>th</sup>, 30<sup>th</sup>, 45<sup>th</sup>, and 60<sup>th</sup> min of re-oxygenation and after the NE application.

### Data analysis

The results are presented as the percentages of the values obtained before the experimental protocol application. All continuous data is presented as a mean ± standard error of the mean (SEM). Two-way analysis of variance (ANOVA) with Holm-Sidack test was used to compare the values from the 5<sup>th</sup> to the 60<sup>th</sup> min of re-oxygenation. The p-values <0.05 were considered statistically significant. Statistical analysis was performed using SigmaPlot software v. 10.0.1.2. (Systat Software Inc., San Jose, USA).

The approval of the local bioethics committee for the use of human tissue was obtained and individual patient consent was waived. All experiments were performed according to the principles stated in the Declaration of Helsinki.

### Results

There were no significant differences in age, sex and pharmacotherapy between the patients from whom the trabeculae were taken and subjected to each experimental protocol.

The application of the NOS blocker – LNMMA at a concentration of 10<sup>-4</sup> M resulted in the decrease of Amax, Slope L and Slope T as compared to control. The co-application

of 10<sup>-4</sup> M morphine with LNMMA, or the application of LNMMA, morphine and SNAP at a concentration of 10<sup>-4</sup> M partially reversed the detrimental effect of LNMMA. All detailed results are depicted in Fig. 2.

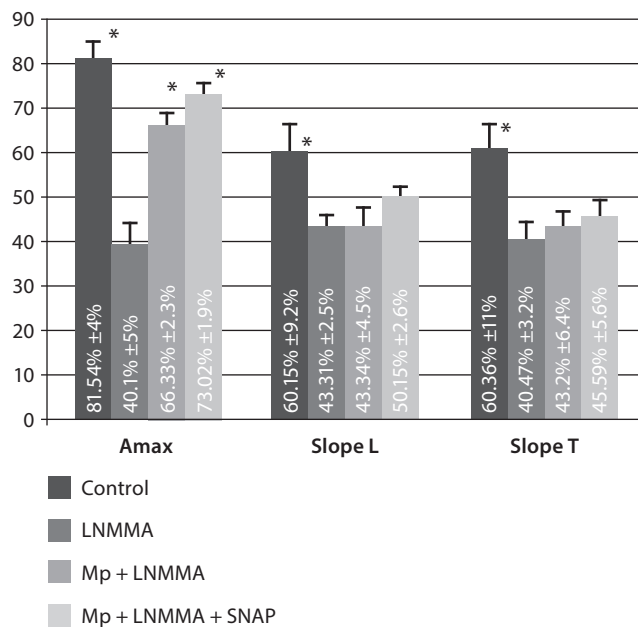


Fig. 2. The effect of *N*-methyl-L-arginine (LNMMA), morphine (Mp) and/or *S*-nitroso-*N*-acetylpenicillamine (SNAP) on the function of the human myocardium during the re-oxygenation period

Control – the protocol with hypoxic conditions only.

### Discussion

Due to the role of NO in cardioprotection, the use of NO donors has been proposed for the prevention of I/R injury and the treatment of chronic cardiovascular diseases.

Accordingly to the earlier obtained data, we reported the cardioprotective effect of morphine on the function of the human heart muscle.<sup>6,7</sup> In this study, we showed that NO plays an important role in intermediating the beneficial influence of opioids. The NOS blockade completely abrogated the effect of morphine. Moreover, the NOS blockade constituted a deleterious factor on the myocardium that is strong enough to outweigh the protective influence of morphine and of the NO donor. To our knowledge, this is the first presentation of the fact that the NO signaling pathway is crucial in the mechanism of protection in relation to the function of the human heart muscle.

Previous studies on the influence of opioids and the NO pathway on I/R injury utilized mainly animal models. We assessed the effect of the co-operation of opioids with NO on the systolic and diastolic function of the hypoxic human myocardium in vitro. Our study was performed on isolated fragments of the human right

atria. For functional studies, due to atrial tissue sampling, it is possible to avoid the influence of confounding factors, like the effect of drugs or the presence of collateral circulation. In this model we did not assess the infarct size, but the differences of contractility as functional consequences of cardiac ischemia.

Morphine is commonly used as an analgetic drug in acute myocardial infarction. This was the first opioid drug shown to be cardioprotective against I/R injury. Opioids also appear to mediate cardioprotective strategies – IPC and POC, brief non-lethal episodes of acute lethal I/R injury – applied respectively before the onset of the re-oxygenation period.<sup>8,9</sup> The beneficial effect of opioids, IPC or POC has been shown in many studies using animal models and clinical trials, although the intracellular mechanism responsible for this phenomena remains not fully understood.<sup>10–12</sup>

It is well-known that NO acts as a modulator of the analgetic effect of morphine and the inhibitors of the NO/cGMP pathway attenuate this effect.<sup>13</sup> Several studies reported that the NO inhalation effectively reduced the infarct size of the heart muscle and improved the cardiac function in porcine and rodent models of I/R injury.<sup>14,15</sup> Recently, it has been shown that remote intrathecal fentanyl preconditioning induces cardioprotective effects in rats via the activation of NOS, and that the NOS inhibitor – N omega-nitro-L-arginine methyl ester (L-NAME) abolished this effect.<sup>16,17</sup> Further studies highlight the role of the increased expression and activity of NOS in delayed protective effects of IPC.<sup>18</sup> POC also increases NOS activity; likewise, the NOS blockade with L-NAME abrogates the beneficial effect of POC.<sup>19</sup>

In our study, the use of the NO-donor, SNAP, improved the systolic and diastolic function of human myocardium in the re-oxygenation period. In a rat model, the administration of SNAP reduced the size of necrosis.<sup>20</sup> Moreover, the use of a substrate for the synthesis of NO, L-arginine, resulted in a reduction in infarct size and improved the cardiac systolic function.<sup>19</sup> Apart from the fact that SNAP releases NO, providing a mediator of intracellular pathways, it has also the ability to inhibit the inducible NOS (iNOS) expression, responsible for the synthesis of the highly reactive peroxy-nitrite (ONOO<sup>-</sup>), preventing damage generated by oxidative stress.<sup>21</sup> Although higher SNAP concentrations did not confer protection, which results from twofold nature of NO.<sup>20</sup> The cardioprotective effect depends on the balance between NO and reactive oxygen species (ROS). ROS released during re-oxygenation lead to the opening of mPTP, which triggers the depolarization of the mitochondrial inner membrane, resulting in the adenosine triphosphate (ATP) depletion, the respiratory chain inhibition and the rupture of the mitochondrial outer membrane. The activation of protein kinases, such as Akt, glycogen synthase kinase 3β (GSK-3β), which consists of the element downstream

of NO/cGMP in the intracellular pathway of opioids, prevents the opening of mPTP.<sup>22</sup> Physiological NO concentration inhibits the mPTP opening, whereas a high concentration of NO favors the creation of ONOO<sup>-</sup> and triggers the mPTP opening related to the formation of disulfide bonds.<sup>23</sup> Furthermore, the transient activation of NOS during reperfusion involves the rapid consumption of L-arginine and tetrahydrobiopterin, causing the production of the superoxide anion instead of NO.<sup>24</sup>

It is feasible that the cooperation of opioids with NO in the cardioprotective effect arises from the common signaling pathway. On the contrary, the double-edge sword effect of NO donors explains the diverging results, which is reflected in the exogenous nitrate tolerance.<sup>24</sup>

## Limitations

The results must be interpreted within the limitations of the methodology. The construction of our experiment assumes a control group derived from the same patient, and the same factors potentially affecting the test. We must note, however, that the simulated ischemic model differs from in vivo conditions. In our experiment, we utilized the buffer, so there were no elements transporting or binding opioids, like peptides. However, the pathophysiological and functional changes that took place in our model of I/R injury are comparable to the change that takes place in in vivo conditions. The instantaneous NO concentration is crucial in many processes, but its evaluation is hard to obtain, which constitutes the limitation of the method.

## Conclusions

In the re-oxygenation period, the blockade of NO synthesis has a deleterious effect on the systolic and diastolic function of the human myocardium.

All protocols were preceded by a stabilization period of 45–60 min. This was followed by 60 min of simulated ischemia (superfusion with the hypoxic, substrate-free Krebs-Henseleit solution and a pacing at 1 Hz) and 60 min of superfusion with the re-oxygenated Krebs-Henseleit solution. In protocols with a pharmacological modification, *N*-methyl-L-arginine at a concentration of 10<sup>-4</sup> M, *S*-nitroso-*N*-acetylpenicillamine at a concentration of 10<sup>-4</sup> M and/or morphine at a concentration of 10<sup>-4</sup> M were used. Control – the protocol with hypoxia conditions only.

Figures present parameters of the contraction as maximal force of a contraction (Amax), the rate of rise of the force of a contraction (Slope L) and the relaxation parameter – the rate of decay of the force of a contraction (Slope T); \* indicates significantly higher values (p < 0.05) vs LNMMA.

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