Acta Physiologica Sinica, August 25, 2004, 56 (4): 503-508
http://www.actaps.com.cn

Research Paper

Medullary ventrolateral nitric oxide mediates the cardiac effect of electroacupuncture at "Neiguan" acupoint on acute myocardial ischemia in rats

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Abstract: Experiments were performed on male Sprague-Dawley (SD) rats anesthetized with a mixture of urethane and chloralose. A rat model of acute myocardial ischemia (AMI) was made by ligation of the left anterior descending branch of the coronary artery (LAD). After the LAD ligation, the ischemia area of the left ventricular wall became somewhat pale immediately. Under a light microscope, the pathological examination revealed that all the cells were swollen and in red color when the cardiac section was stained with hematoxylin basic fuchsin picric acid (HBFP), which indicated a typical change in the myocardial ischemia. In the AMI model, it was found that cardiac functions were markedly attenuated, such as decreases in the heart rate (HR), mean arterial pressure (MAP), left ventricular systolic pressure (LVSP), maximal rate for left ventricular pressure rising and declining ($\pm dp/dt_{max}$), velocity of contractile element (V_{CE}) and total area of cardiac force loop (L_0), and an increase in the left ventricular end diastolic pressure (LVEDP). In such AMI rats, application of electroacupuncture (EA) at "Neiguan" acupoints (Pe 6) for 20 min could obviously improve the above-mentioned cardiac functions. After microinjection of nitro-*L*-arginine (*L*-NNA), an inhibitor of nitric oxide synthase (NOS), was made into the rostral ventrolateral medulla (RVLM), the curative effect of EA on myocardial ischemia was reduced significantly or abolished, while after microinjection of normal saline of the same volume was made into the RVLM, the improving effect of EA remained. These results suggest that the effect of EA on myocardial ischemia is possibly mediated by the nitric oxide (NO) in the RVLM.

Key words: acupuncture; acute myocardial ischemia; cardiac function; nitric oxide; rostral ventrolateral medulla

延髓腹外侧一氧化氮介导电针内关对急性心肌缺血大鼠心功能的作用

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摘 要: 实验在以乌拉坦和氯醛糖混合麻醉的雄性 SD 大鼠上进行。结扎左冠状动脉前降支以建立急性心肌缺血(AMI)动物模型。病理学检查显示该模型具有典型的心肌缺血改变。功能学改变包括心率(HR)减慢、平均动脉压(MAP)降低, 以及心功能减弱, 如左室舒张末压(LVEDP)增大, 左室收缩压(LVSP)、左室压变化最大速率($\pm dp/dt$)、左室收缩成分缩短速度(V_{CE})、心力环总面积(L_0)等均明显减小。电针 AMI 大鼠的内关穴位 20 min, 可使其 HR、MAP、LVEDP、LVSP、$\pm dp/dt$、V_{CE} 和 L_0 等均明显改善。若电针前于延髓头端腹外侧区(RVLM)微量注射一氧化氮合酶(NOS)抑制剂 *L*-NNA (0.1 mmol/L, 0.1 μl), 除 HR 和 MAP 外, 电针改善 AMI 心功能的其余各项指标均减弱或被取消, 而以等量的生理盐水取代 *L*-NNA 被注入 RVLM 时, 则不能影响 EA 对 AMI 各项心功能指标的改善作用。以上结果提示电针内关改善 AMI 的作用由 RVLM 的一氧化氮(NO)所介导。

关键词: 针刺; 急性心肌缺血; 心功能; 一氧化氮; 延髓头端腹外侧区
中图分类号: Q463

Received 2004-01-12 Accepted 2004-03-23

This work was supported by the National Natural Scientific Foundation of China (30340071) and the Health Department of Shanghai Government (2000-3).

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Cardiovascular diseases, such as hypertension, acute myocardial ischemia (AMI) and coronary heart disease, are main diseases harmful to human health. The morbidity and mortality of these diseases are going up gradually in recent years. Acupuncture has been used to treat patients with various kinds of diseases for over two thousand years in China. However, its physiological basis remains undefined. Since the 1980s, our laboratory of the Department of Physiology at Shanghai Medical University has done a series of work demonstrating that acupuncture has curative effects on experimental hypertension, arrhythmias, hypotension and shock[1,2]. Longhurst also proved that acupuncture is effective in improving myocardial ischemia[3]. Many researchers considered that the peripheral and central nervous system is involved in the mechanism of modulatory effect of acupuncture on abnormal cardiovascular functions[1-3]. Further studies have shown that some neurotransmitters in some cardiovascular centers play a key role in acupuncture mechanisms. Xie *et al*. reported that stimulation of the deep peroneal nerve (DPN) underneath the "Zusanli" acupoint (St 36) with a low current and low frequency could attenuate the hypertensive effect induced by the stress stimuli of electrical foot-shock combined with noises, and the attenuation (i.e. depressor effect) of DPN stimulation was blocked by microinjection of naloxone, a nonselective antagonist of opioid receptor, into the rostral ventrolateral medulla (RVLM)[4]. Li *et al*. reported that electroacupuncture (EA) at "Zusanli" acupoint could improve the cardiorascalar functions during cardiac ischemia and reverse the stress-induced hypertension; these effects were also blocked by microinjection of *L*-NNA, an inhibitor of nitric oxide synthase (NOS), into the ventral periaqueductal gray (VPAG) in the midbrain[5]. It is well known that NO is an important molecule for signal transmission in the central nervous system, and widely distributed in the cardiovascular center, including the RVLM[6]. The present study was designed to investigate whether medullary ventrolateral NO is involved in the mechanism of EA at "Neiguan" acupoint (Pe 6) on curing the AMI in rat model.

1 MATERIALS AND METHODS

1.1 *Animal preparations.* Experiments were performed on male Sprague-Dawley (SD) rats (250~300 g) anesthetized with a mixture of urethane and chloralose (700 mg/kg and 35 mg/kg, i.p.), paralyzed by Flaxedil (5 mg/kg, i.v. for the first dose, supplemental dose of 0.5~1.0 mg/kg at 2~2.5 h interval) and ventilated artificially with a respirator (TKR-200C, Jiangxi Teli Anesthesia & Instrument, Nanchang, Jiangxi) with a volume of 3 ml each breath and 80 breaths per minute. Arterial blood pH was maintained at 7.35~7.45. Arterial blood pH, PCO_2 and PO_2 were monitored by means of a blood gas analyzer (Medica Easy Blood, Medica Corp., USA) of Zhongshan Hospital. An arterial catheter was inserted into the femoral artery for measurement of blood pressure (BP), and another catheter was inserted into the left ventricle of the heart via the left carotid artery for recording intraventricular pressure, then the two catheters were connected to a bioelectric signals processing system (Model SMUP-A, Department of Physiology, Shanghai Medical University) via a separate pressure transducer, from which the cardiac function data were analyzed and obtained. The indices of cardiac functions included the heart rate (HR), mean arterial pressure (MAP), left ventricular end diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP), maximal rate for left ventricular pressure rising and declining ($\pm dp/dt_{max}$), velocity of contractile element (V_{CE}) and total area of cardiac force loop (L_0). During the experiment, the rectal temperature of the animal was measured and kept at 37.5±0.5ºC.

1.2 *Acute myocardial ischemia (AMI) model*. Thoracotomy was performed at the third and forth left intercostal space. The left anterior descending branch of the coronary artery (LAD) was ligated at the level of 2~3 mm below the initial part of the aorta. The ischemia area of the left ventricular wall became somewhat pale immediately after the ligation. After each experiment was finished, the rat was killed, the heart was cut and fixed in a 10% formation solution for 7 d. Then the paraffin sections of myocardial tissue were made and stained with hematoxylin basic fuchsin picric acid (HBFP)[7] for pathological examination of myocardial ischemia.

1.3 *Application of electroacupuncture*. Two stainless steel needles were inserted into both "Neiguan" acupoints, corresponding to that of human beings, which are located in the interosseal muscles between the radius and the ulna of the distal medial thoracic limb at the level of 3 mm superior to the wrist joint in rats. The electric impulses were derived from a medical stimulator (G6805-2, Shanghai Medical Apparatus) at a frequency of 4 to 20 Hz alternated sequentially, 0.5 ms duration and at an intensity just strong enough to elicit slight twitches of the foot. Application of EA was continued for 20 min each time.

1.4 *Brain microinjection.* The head of the rat was fixed on a stereotaxic apparatus flexed to an angle of about 45º, the occipital bone was removed to expose the fourth ventricle,

and its floor was kept at horizontal level. A stainless steel cannula with its outside diameter of 0.2 mm was inserted into the right RVLM (1.5~2.5 mm ahead the obex, 1.5~2.0 mm right to the central line and 6.6~7.0 mm depth from the dorsal surface of the cerebellum) according to the atlas of Paxinos and Watson[8]. Nitro-*L*-Arginine (*L*-NNA, 0.1 mmol/L, Sigma), an inhibitor of NOS, or normal saline (NS, as a control), was microinjected into the RVLM in a volume of 0.1 μl over a period of 1 min through the inserted cannula. In each experiment, the animal took a rest for 2 h after the surgical operation, and then the microinjection and observation began. At the end of each experiment, the microinjection sites in the RVLM were identified by injection of Pontamine Sky Blue dye (0.1 μl). Then the animal was killed, and the brain was removed and fixed in a 10% formation solution for 7 d. Brain rozen cross-sections (30 μm) were made and stained with neutral red to identify the microinjection sites (Fig. 1).

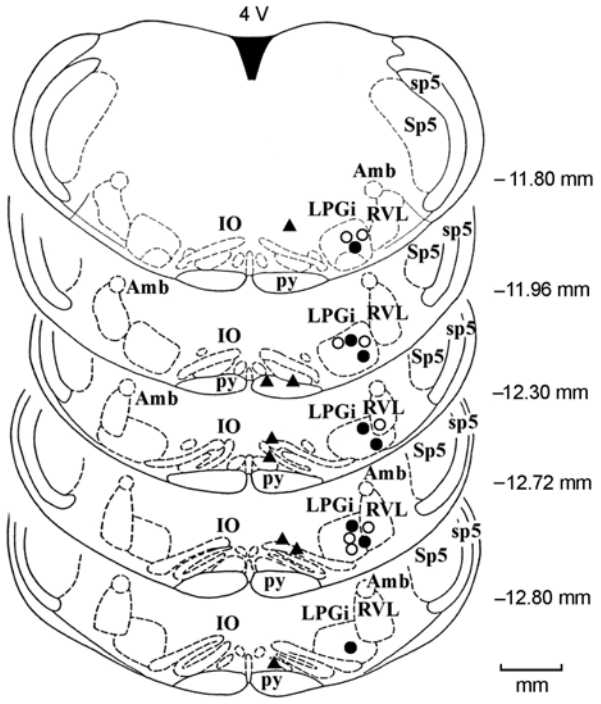

Fig. 1. Location of microinjection of drugs. In each cross section, the dots and circles indicate the sites of microinjection of *L*-NNA (effective) and NS (ineffective) into the RVLM, respectively, and the black triangles indicate the sites of microinjection of *L*-NNA out of the RVLM (ineffective). The negative digits in a unit of millimeter indicate the position of each cross section behind the bregma. Amb, ambiguous nucleus; 4 V, the 4th ventricle; IO, inferior olive; LPGi, lateral paragigantocellular nucleus; py, pyramidal tract; RVL, rostroventrolateral nucleus; Sp5, spinal trigeminal tract nucleus; sp5, spinal trigeminal tract.

1.5 *Statistical analysis*. Experimental data were expressed as mean±SD. Statistical analysis was performed by *t* test. It was considered that the difference was significant when the *P* value was less than 0.05.

2 RESULTS

2.1 Cardiac histological changes in the AMI model

During our making the AMI model, the ischemia area of the left ventricular wall became somewhat pale immediately after ligating the LAD. The pathological examination was made under a light microscope, the section of ischemic myocardia stained with HBFP revealed that all cells were swollen and in red color (according to the reference 7, the degree of red staining with HBFP was "+++"), which provided the strong evidence of typical myocardial ischemia in the early stage (Fig. 2*A*); while the normal myocardia stained with HBFP were yellow stained (according to the

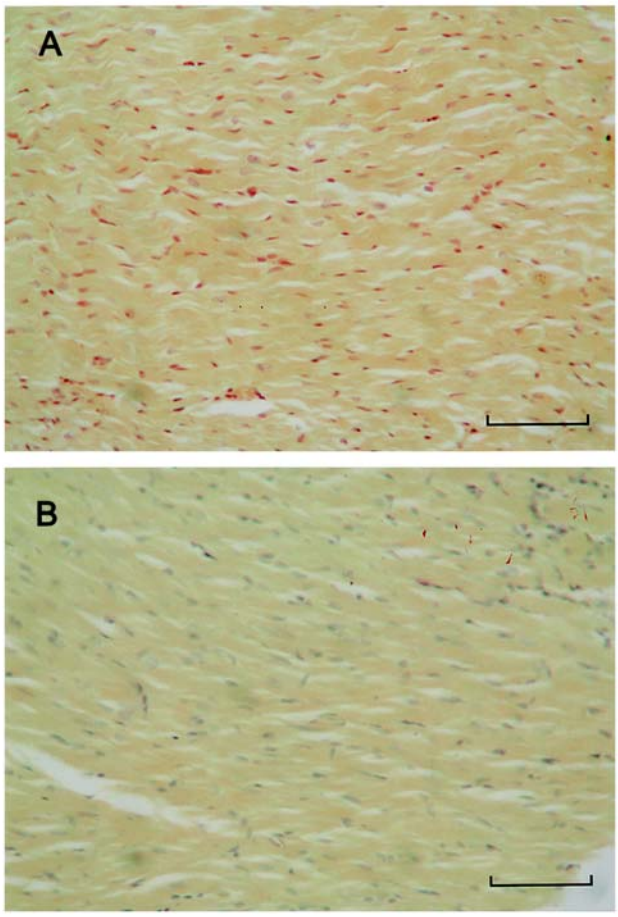

Fig. 2. *A*: Histological appearance of ischemic myocardia in a paraffin section stained with HBFP. Myocardia were swollen and stained red. *B*: Histological appearance of normal myocardia in a paraffin section stained with HBFP. Myocardia were stained yellow and not swollen. The magnitude scales in both *A* and *B* indicate 50 μm length.

reference 7, the degree of staining with HBFP was "–") and no swelled changes (Fig. 2*B*).

2.2 Cardiac functional changes of the AMI model

In the AMI model (n=8), almost all the indices of the cardiac functions, including HR, MAP, LVSP, ±*dp/dt*, V_{CE} and L_0, decreased significantly (P<0.05 or P<0.01, Table 1) (Fig. 3, 4) as compared with those of the control group, while LVEDP increased significantly (P<0.01, Table 1, Figs. 3, 4).

2.3 Effects of EA on cardiac functions of the AMI model

On the AMI rat model (n=8), EA at "Neiguan" acupoint was applied for 20 min, the frequency was of 4 to 20 Hz alternated sequentially, and the duration and intensity was 0.5 ms and just strong enough to elicit slight twitches of the foot, respectively. Such treatment could obviously reverse the above indices of cardiac functions of the AMI model (P<0.05 or P<0.01, Table 1), which indicated that the EA could improve the cardiac function of the animal suffering from the AMI. To test the specificity of the "Neiguan" acupoint, EA with the same parameters was applied nearby the acupoint, but the reversal of cardiac functional indices of the AMI model could not be seen.

Table 1. HR, MAP and other indices of cardiac functions (mean±SD, n=8) in the groups of control, AMI and AMI+EA, respectively

	Control	AMI	AMI+EA
HR (beats/min)	419 ± 38	390 ± 10*	412 ± 13$^{\wedge\wedge}$
MAP (mmHg)	104 ± 7	73 ± 4**	85 ± 4$^{\wedge\wedge}$
LVEDP (mmHg)	–9 ± 2	–6 ± 2**	–9 ± 3$^{\wedge}$
LVSP (mmHg)	174 ± 11	128 ± 9**	160 ± 3$^{\wedge\wedge}$
+*dp/dt* (mmHg/s)	13430 ± 3691	6544 ± 802**	13452 ± 1078$^{\wedge\wedge}$
–*dp/dt* (mmHg/s)	–8184 ± 2210	–5544 ± 802**	– 7232 ± 787$^{\wedge\wedge}$
V_{CE} (s^{-1})	4.0 ± 0.20	3.7 ± 0.21**	4.04 ± 0.12$^{\wedge\wedge}$
L_0 (CFU)	242 ± 64	97 ± 23**	173 ± 25$^{\wedge\wedge}$

$^{*}P$<0.05, $^{**}P$<0.01 *vs* control group, $^{\wedge}P$<0.05, $^{\wedge\wedge}P$<0.01 *vs* AMI group.

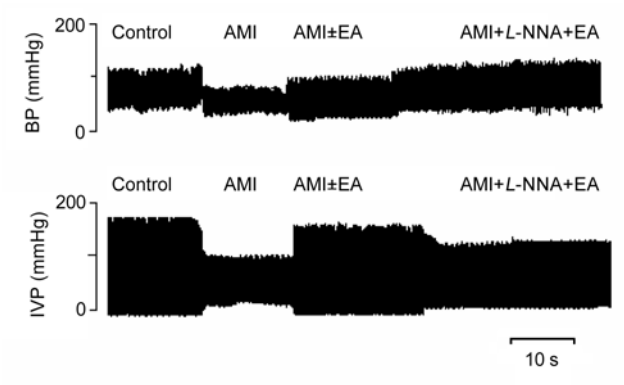

Fig. 3. Blood pressure (BP) and intraventricular pressure (IVP) records during baseline (control level), AMI, application of EA on AMI (AMI+EA) and microinjection of *L*-NNA into the RVLM prior to EA on AMI (AMI+*L*-NNA+EA).

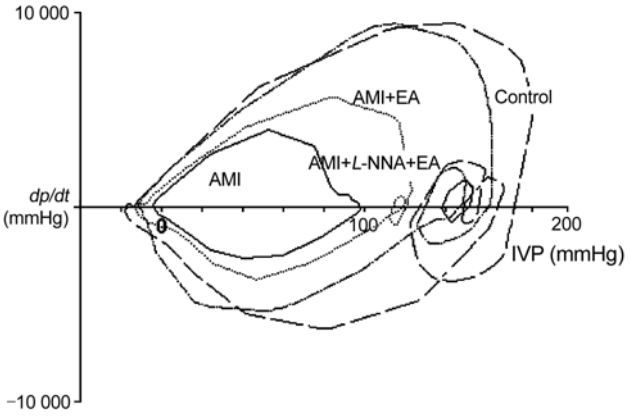

Fig. 4. Total area of cardiac force loop (L_0) of groups of control, AMI, AMI+EA and AMI+*L*-NNA+EA, respectively.

2.4 Medullary ventrolateral NO was involved in the effect of EA on the AMI

In 8 rat models with AMI, *L*-NNA (0.1 mmol/L, 0.1 μl) was microinjected into the right RVLM prior to the application of EA, which significantly reduced or abolished the improvement effect (except HR and MAP) of EA on myocardial ischemia (P<0.05 or P<0.01, Table 2), while normal saline (NS, 0.1 μl), instead of *L*-NNA, was microinjected into the RVLM, the improvement effect of EA was

Table 2. HR, MAP and other indices of cardiac functions (mean ± SD, n=8) in the groups of microinjection of *L*-NNA or NS (as a control) into the RVLM, respectively, prior to application of EA

	AMI+EA	AMI+NS+EA	AMI+*L*-NNA+EA
HR (beats/min)	412 ± 13	405 ± 12	410 ± 12
MAP (mmHg)	85 ± 4	84 ± 3	88 ± 3
LVEDP (mmHg)	−9 ± 3	–10 ± 3	–2 ± 4**
LVSP (mmHg)	160 ± 3	162 ± 3	137 ± 2**
+*dp/dt* (mmHg/s)	13452 ± 1078	12752 ± 988	8947 ± 976**
–*dp/dt* (mmHg/s)	–7232 ± 787	–7398 ± 776	–6318 ± 752*
V_{CE} (s^{-1})	4.04 ± 0.12	4.05 ± 0.10	3.66 ± 0.11**
L_0 (CFU)	173 ± 25	168 ± 24	124 ± 23**

*P<0.05, ** P<0.01 *vs* AMI+NS+EA group.

not altered (P>0.05).

3 DISCUSSION

3.1 AMI model

In the present study, we made the AMI model by a classical method, i.e. ligation of the LAD[9]. From pathological and functional observation, indices of this model indicated that the AMI model was successfully made and the myocardial ischemia was actually existence. The decrement in MAP, LVSP, ±*dp*/*dt*, V_{CE} and L_0 suggested that the myocardial contractility was injured due to the insufficient blood supplies. The decrement in HR and increment in LVDEP are also unfavorable for the heart as a pump.

In our experiments, actually, the acute myocardial damage was not very serious, just like cardiac infarction during the early stage, so it was difficult to estimate the myocardial damage by hematoxylin-eosin (HE) stained histological section, while the method of HBFP staining is appropriate for the diagnosis of AMI. The ischemia myocardium in the early stage is highly sensitive to such staining and has certain criterion[7]. Myocardia are swollen and red stained which can be distinguished into several degrees in the positive section stained with HBFP. The degree of the myocardial damage in our model was "+++".

3.2 Curative effect and its mechanism of acupuncture on AMI model

Many studies have confirmed that acupuncture or EA at "Neiguan" acupoint could reduce the coronary flow resistance, promote the establishment of collateral circulations, so it can increase blood supplies in the ischemic area. EA at "Neiguan" acupoint could also reduced oxygen consumption and enhance the myocardial contractility in the ischemic area, so that it contributes to the recovery of the cardiac functions. In addition, EA at "Neiguan" acupoint could raise the antioxidation ability of the serum and myocardial tissue, so as to stop or lessen the further injury of lipoid hyperoxidation[10, 11].

The present study showed that EA at "Neiguan" acupoint could obviously improve the cardiac functions in the AMI model, and the improvement effect could be blocked by microinjection of *L*-NNA, an inhibitor of NOS, into the RVLM, which suggests that the effect is mediated by NO in the cardiovascular center. In our previous work, we observed that microinjection of *L*-arginine, a precursor of NO, into the RVLM could cause a depressor effect and a decrease in the pressor response induced by electrical stimulation of the dorsal periacqueductal gray (DPAG), whereas microinjection of *L*-NMMA, an inhibitor of NOS, into the RVLM could elicit a pressor effect and an increase in the pressor response induced by DPAG stimulation, which indicates that medullary ventrolateral NO plays an inhibitory role on sympathetic efflux, reduces the cardiac oxygen consumption and is benefit to cardiac functions[12]. Yang and He reported that the discharge rate of the RVLM neurons decreased in response to intravenous injection (i.v.) of NO donors sodium nitroprusside (SNP) or 3-morpholinosydnonimine (SIN-1), but increased in response to i.v. of NOS inhibitor *L*-NNA[13]. Yang and He also observed that microinjection of *L*-NNA or SNP into the RVLM could change the blood pressure and heart rate which were similar to our observation[14]. Many reports described the distribution of NOS in the ventrolateral medulla that are involved in cardiovascular regulation[15,16].

It is well known that the RVLM is an important area of the cardiovascular center. The neurons in this area receive inputs from hypothalamic and midbrain defense areas, other cardiovascular centers, and some afferent impulses from

baroreceptor, chemoreceptor, cardiopulmonary receptor, somatic and splanchnic nerves, acupoints, etc., and send outputs descending to the preganglionic sympathetic neurons in the intermediolateral column (IML) of the spinal cord. Much cardiovascular regulatory information integrates here. So the cardiovascular neurons in the RVLM are considered very important to control the peripheral sympathetic tone and cardiovascular activities[1, 2].

There is good evidence that the NO and NOS distribute plentifully in the RVLM and contribute to cardiovascular regulation[6]. Activation of NO in the RVLM could reduce the sympathetic outflow, whereas inhibition of NO in the RVLM could increase the sympathetic outflow, induce the incidence of cardiovascular diseases and aggravate the state of these diseases. The present study showed that the curative effect of EA could be blocked by inhibition of NO activities in the RVLM, which suggests that EA exerts its curative effect through reduction of sympathetic outflow derived from the cardiovascular center. But the detailed mechanism requires further investigations.

Acknowledgement:

We thank QIAN Yuan and DING Ying-Jong for their expert technical assistance.

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