Blockade of spinal nerves inhibits expression of neural growth factor in the myocardium at an early stage of acute myocardial infarction in rats

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Editor’s key points

- Neural growth factor (NGF) is important for nerve regeneration after injury to the heart.
- NGF was up-regulated after coronary artery occlusion in rats.
- Blocking the spinal nerves to the heart with local anaesthetics abolished the NGF up-regulation.
- This may be important in patients.

Background. Neural growth factor (NGF) is required for healing and sprouting of cardiac sympathetic and sensory nerves and plays important roles in cardiac protection, sustaining cardiac function and regeneration in ischaemic heart disease. The overexpression or lack of the NGF could be harmful to the heart. In this study, we examined the role of spinal nerves in the modulation of expression of the NGF in the myocardium at risk of ischaemia soon after acute myocardial infarction in rats.

Methods. Coronary artery occlusion (CAO) was carried out in anaesthetized rats with and without preconditioning of blockade of the spinal nerves. The expression of the NGF protein and mRNA in the myocardium at risk of ischaemia was examined using immunohistochemical assay, enzyme-linked immunosorbent assay, and real-time quantitative reverse transcription polymerase chain reaction assay.

Results. In the left ventricle, immunoreactive cells and fibre-like structures were mainly located in the myocardium and in the epicardium. The NGF protein expression was increased by two-fold in the myocardium at risk of ischaemia during the 60 min of CAO, while the NGF mRNA was up-regulated three-fold, at 360 min after acute myocardial infarction. The blockade of the spinal nerves completely abolished the up-regulation of the NGF in the myocardium (P<0.05).

Conclusions. The spinal nerves innervating the heart may play an important role in sustaining the up-regulation of the NGF in the myocardium early after acute myocardial infarction, an effect which can be inhibited by the blockade of these nerves.

Keywords: epidural anaesthesia; nerve growth factor, acute myocardial infarction; spinal nerves

Accepted for publication: 8 March 2012

Nerve growth factor (NGF) is a critical neurotrophic factor required for the development, growth, and survival of peripheral autonomic and sensory neurones. However, in adults, the NGF plays a key role in the regeneration of the nerves after damage. The concentration of the NGF in tissues determines the density of innervation of sympathetic and sensory nerves. Innervation of cardiac nerves is an important issue in heart transplantation, post-infarction remodelling, and cardiac arrhythmias. However, unbalanced regeneration of the cardiac sympathetic nerves is associated with NGF activity, which is potentially responsible for the lethal cardiac arrhythmias in the process of myocardial remodelling after acute myocardial infarction (AMI). Substantial evidence indicates that increased local NGF expression may also play an important role in cardiac protection, sustaining cardiac function, and myocyte regeneration in ischaemic heart disease. Therefore understanding the mechanism by which the production and release of the NGF in the myocardium is modulated during acute myocardial infarction is important to preserve the beneficial effects of the NGF while avoiding harmful consequences.

Previous studies indicated that the sympathetic nerves or neurotransmitters may influence the expression of the NGF, implying a potential interruption of NGF expression in the heart by the blockade of spinal nerves, which mainly consist of efferent sympathetic and afferent sensory nerves. This can be achieved during anaesthesia for surgery and postoperative analgesia. The aim of this study was to examine the effects of the blockade of spinal nerves on the expression of the NGF in the myocardium of rats at risk of ischaemia using a rat model of acute myocardial infarction induced by permanent coronary artery occlusion (CAO). The blockade of
spinal nerves was carried out by epidural anaesthesia at the upper thoracic level, and the expression of NGF proteins and mRNA in the myocardium was analysed using fluorescence immunohistochemical (FIHC) assay, real-time quantitative reverse transcription polymerase chain reaction (qRT–PCR), and enzyme-linked immunosorbent assay (ELISA).

Methods

Protocol

The experiments were approved by the Institutional Animal Care and Use Committee of Shanxi Medical University and conformed to the guidelines for the care and use of laboratory animals (National Institute of Health Guide for the Care and Use of Laboratory Animals, NIH Publications No. 80-23, revised 1996). Healthy male Sprague–Dawley rats weighing 260–280 g (Shanxi Medical University Experimental Animal Laboratory, Shanxi, China) were used for the experiments. The animals were allowed to acclimatize to the laboratory environment for 2 weeks. All surgical procedures were performed under general anaesthesia with urethane (25%, 1.2 g kg⁻¹, i.p.). The adequate depth of anaesthesia was ascertained by the observation of the changes in the size of pupils, and the depth and the pattern of respiration upon noiceptive stimulation. A continuous administration of 0.9% physiological saline (1 ml h⁻¹) was maintained during the experiment.

To study the temporal variation and the mechanism of NGF expression in the myocardium during ischaemia of the left ventricle after CAO, we quantitatively analysed the expression of the NGF protein at 15, 30, 60, and 360 min of CAO using ELISA, and mRNA expression at 15 and 360 min of CAO using qRT–PCR assays.

After successful implantation of an epidural catheter, 132 male Sprague–Dawley rats were randomly divided into three groups: the sham surgery group (sham, n=36), the CAO group (n=36), and the group of thoracic epidural anaesthesia plus the CAO group (EA, n=36). Each group was further divided into four subgroups, according to the time of observation after CAO: 15, 30, 60, and 360 min. In each subgroup, six animals were used for ELISA measurements (n=6) and three for FIHC (n=3) at the scheduled time. Four animals were, respectively, assigned to each of the two subgroups for qRT–PCR assay at 15 min (n=4) and 360 min (n=4) after CAO. The animals in the EA group were injected with 1% ropivacaine in the epidural space of the thoracic segments, and the animals in the other groups were given 0.9% saline.

Epidural catheterization

The procedure for epidural catheterization was the same as we have reported previously.⁹ Briefly, after making a small incision through the occipitoaxial ligament, PE-10 tubing was inserted caudally into the epidural space reaching the level of the second or the third thoracic segment (T2–T3) of the rats. After recovery from the surgery and anaesthesia for 48 h, animals exhibiting any sign of neurological impairment were excluded from the study. Successful implantation of the epidural catheter was ascertained by the detection of reversible segmental loss of response to noxious stimulation in thoracic segments (T1 – T8) without motor disturbance in hind limbs after injection of 20 μl of 1% lidocaine through the catheter.

Acute myocardial ischaemic model

The acute myocardial infarction model was prepared as we reported previously.⁹ ¹⁰ Briefly, the pericardium was opened through an incision in the left fourth intercostal space under general anaesthesia and mechanical ventilation. A permanent ligation of the left anterior descending branch of the coronary artery was performed. Sham-operated rats underwent the same surgery as described above except without the ligation procedure.

The CAO was carried out 15 min after epidural injection of either 20 μl of 1% ropivacaine for the animals in the EA group or the same volume of 0.9% saline for the rats in the CAO and sham surgery groups. The arterial pressures and heart rate were monitored via a cannula inserted in the left carotid artery of the animals, and CAO was confirmed by the changes in the ECG and by autopsy.

Definition of the myocardium at risk of ischaemia

To identify the myocardium at risk of ischaemia, 1.5 ml of 1.0% Evans blue (Sigma-Aldrich, St Louis, MO, USA) was injected into the caudal vein, dyeing the perfused myocardium with a blue colour. The myocardium not stained was defined as the myocardium at risk of ischaemia (Fig. 1). Samples of the myocardium at risk were collected for further processing from animals in CAO and EA groups and from a matching position of the hearts of the animals in the sham surgery group.

FIHC assay

The hearts were removed, processed, embedded in optimal cutting temperature medium (OCT, Bioprotfolio, Dorset, UK), and sectioned (8 μm) using a cryostat (Leica CM 1850, Nussloch, Germany). The FIHC method used is described in Supplementary material online.

Quantitative reverse transcription polymerase chain reaction

Samples were collected from the myocardium at risk of ischaemia at 15 and 360 min after CAO or sham surgery. The qRT–PCR assay was performed according to the manufacturer’s protocol (as described in Supplementary material online), as reported previously.¹⁰

Enzyme-linked immunosorbent assay

The concentration of the NGF in the myocardium was determined using NGF Emax Immuno-Assay kit (Promega, WI, USA) according to the manufacturer’s instructions, measured at 450 nm with a SpectraMax-Plus Microplate spectrophotometer (Thermo Electron Corporation, MA, USA). Data are presented as picograms per gram [pg g⁻¹ total protein (TP)]. All samples were assayed in duplicate.
Fig 1. Identification of the myocardium at risk of ischaemia and changes in ECG during AMI. (a) The red colour indicates the myocardium at risk. (b) ECG before and at 10, 15, 30, 180, and 360 min after CAO, showing the elevation of ST-segment and arrhythmia during AMI. A representative experiment from a single animal. CAO, coronary artery occlusion; AMI, acute myocardial infarction.
Statistical analysis

Values are presented as mean and standard deviation (SD). The Student t-test and one-way ANOVA with post hoc Bonferroni’s test were performed to analyse the differences between groups. A P-value of <0.05 was considered statistically significant.

Results

Eleven rats were excluded from the study: six rats died (two in the CAO group and four in the EA group) before the end of the observation, four had neural impairment after the implantation of the epidural catheter, and one had a blocked epidural catheter. The data reported here were from the 132 animals with successful implantation of the epidural catheter.

Changes in ECG and haemodynamics

An elevation of ST-segment was detected immediately after the onset of CAO and remained throughout the CAO, and arrhythmia was observed in the early period of the CAO (Fig. 1), indicating an immediate response to the acute myocardial infarction. Reductions in systolic arterial pressure (4%), diastolic arterial pressure (13%), and heart rate (5%) were seen at 15 min of the epidural anaesthesia (Fig. 2).

NGF expression

The immunoreactive NGF was visualized in the cells located in the epicardium (Fig. 3A–C) and scattered among the ventricular myocytes (Fig. 3D–I) and was in the fibre-like structures in the myocardium (Fig. 3G–I) of animals in the sham surgery and CAO groups.

The NGF protein in the myocardium at risk increased throughout the first 60 min of CAO, reaching the statistical significance level 30 min (1.5 × sham value, P=0.05) and peaked at 60 min of CAO (2.3 × sham control, P=0.002). This suggested that the increase in the NGF was associated with cessation of blood flow in the myocardium. However, by 360 min after CAO, the expression of the NGF was not different from the control (Fig. 4).

A significant increase in NGF mRNA expression was observed at 360 min in the CAO group (3.1 × sham, P=0.005). However, no difference in mRNA expression was seen 15 min after CAO (Fig. 5).

Effects of spinal nerve blockade on NGF expression

Significantly decreased NGF protein expression was seen at 15, 30, and 60 min after CAO, but mRNA was unchanged at 15 min (Fig. 4) in the animals pretreated with epidural anaesthesia, compared with the CAO animals without epidural anaesthesia, suggesting that NGF expression may depend on the neural activity of spinal nerves. Furthermore, mRNA

Fig 2 Changes in arterial pressure and heart rate. (A) Arterial pressure (mm Hg). (B) Heart rate (beats min⁻¹). Arrows indicate the start of epidural anaesthesia (E), the thoracotomy (T), and the CAO (C).
**Fig 3** The location of NGF immunoreactive cells and fibres in the myocardium. In the myocardium at risk of ischaemia, NGF immunoreactive cells were mainly located in the epicardium (Ep, A–C) and the myocardium (D–F). The NGF immunoreactive fibre-like structures (G–I) were observed in the myocardium. The arrows indicate the origins of the insets in the images (representative experiment from three animals). Bars are 500 μm in (A and D), 200 μm in (a), 100 μm in (i), and 50 μm in (c, e–g, and i).

**Fig 4** Changes in the NGF protein in the ischaemic myocardium in sham surgery (sham), CAO, and epidural anaesthesia plus CAO (EA) groups at 15, 30, 60, and 360 min after CAO. S1: P=0.027, CAO vs EA; S2: P=0.05, sham vs CAO; S3: P=0.0001, CAO vs EA; S4: P=0.002, sham vs CAO; S5: P=0.0001, CAO vs EA; NS1: P=0.132, sham vs CAO; NS2: P=1.0, sham vs CAO; NS3: P=1.0, CAO vs EA. TP, total protein.

**Fig 5** Expression of the NGF mRNA in the ischaemic myocardium in sham surgery (sham), CAO, and epidural anaesthesia plus CAO (EA) groups at 15 and 360 min after CAO. S1: P=0.005, sham vs CAO; S2: P=0.048, CAO vs EA; NS1: P=0.799, sham vs CAO; NS2: P=0.323, CAO vs EA.
expression was significantly up-regulated, by up to 1.6-fold ($P=0.048$) of that in the CAO animals, at 360 min of the CAO (Fig. 5) in the EA group, while the NGF protein was not different from the control (Fig. 4).

**Discussion**

To investigate the profile of early changes in the NGF 6 h after acute myocardial infarction, we used a rat model which has been proved to be reproducible and consistent, producing an area of $\sim 43\%$ at risk of ischaemia with an infarct area of 26% of the ventricular myocardium after 6 h of CAO. Here, we found that acute myocardial infarction caused increases in the NGF in the myocardium at risk of ischaemia throughout the 60 min of occlusion. A delayed up-regulation of the NGF mRNA was detected 360 min after myocardial infarction. The up-regulation of the NGF occurred earlier than previously reported, where the elevation of the NGF protein but not the corresponding mRNA at 3.5 h after acute myocardial infarction and increased NGF protein and mRNA 3 day post-myocardial infarction in the myocardium were reported. The discrepancy in the response of alterations of the NGF protein and mRNA in the myocardium may indicate that the increase in the NGF early after CAO was mainly via a post-transcriptional process.

The NGF is expressed in normal hearts, and its synthesis and release could be altered under pathological conditions, including acute myocardial infarction. Multicellular sources of the NGF in the myocardium, including within cardiomyocytes, endothelial cells, fibroblasts, cardiac ganglion cells, neutrophils, and macrophages, have been reported. Here, we found that the immunoreactive cells were mainly located in the epicardium and scattered in the myocardium, in contrast to a previous study where clustered immunoreactive cells in the cardiac ganglia were seen. The cells may contribute to the early increase in the NGF in the ischaemic myocardium. However, the increase in the NGF was unlikely to be as a result of assembling the circulating NGF in the myocardium at risk of ischaemia, since the coronary artery was ligated throughout the experiment. The decline in the NGF protein 360 min after the onset of CAO, observed in this study, may be a result of a re-balance between the production and output of NGF, from and out of the myocardium. The NGF could be transported from the myocardium to the neurones located in the sympathetic stellate ganglia and dorsal root ganglia by axonal transportation mechanisms.

The observation of NGF immunoreactive fibre-like structures in the myocardium may serve as supporting evidence. The precise mechanism for the increase in the NGF in the myocardium after the CAO remains unknown. However, anaerobic metabolism in the ischaemic myocardium might initiate the mechanism by which NGF production was promoted in the myocardium, while the neural activities of the spinal efferent and afferent nerves were activated. In a previous report, we used the same experimental model and showed that epidural anaesthesia at the same level resulted in a reduction in myocyte apoptosis and caspase-3 activity. Here, we observed that the blockade of spinal nerves attenuated increased expression of the NGF in the myocardium induced by acute myocardial infarction, which may suggest an involvement of spinal nerves in the modulation of the NGF in the heart.

The findings may suggest that neural mechanisms have an important role in sustaining the expression of the NGF in the myocardium at risk of ischaemia early after acute myocardial infarction. The immediate increase in the NGF after the onset of CAO might be directly enhanced by the neural modulation of spinal nerves. Spinal nerve blockade may directly affect the transduction of cardiac sympathetic nerves and cardiac innervating sensory afferents, and indirectly affect the functions of cardiac ganglion neurones, including the parasympathetic ganglion cells. At this stage, we are unable to confirm that the increase in the NGF is related to sympathetic nerves, or indeed, to rule out the parasympathetic drives (ability to get things done) in the up-regulation of the NGF, although previous reports showed that sympathetic innervation promotes neuronal NGF synthesis from cardiac ganglia. In addition, norepinephrine, a main sympathetic transmitter, was able to reduce the production of the NGF from cardiomyocytes via $\alpha$-adrenoceptor. The findings may suggest that sympathetic activity may have multiple roles in terms of the different cellular sources of myocardium. Although the mechanism of the neural modulation of NGF expression by spinal nerves is not yet clear, our findings suggest that spinal nerves participate in the heart in acute myocardial infarction, possibly via post-transcriptional mechanism. Further study is needed to clarify the individual role of the spinal nerves, mainly the sympathetic efferent and sensory afferent.

Activation of the genes (Fig. 5) and synthesis of the NGF in the myocardium may be initiated later after myocardial infarction, via a feedback mechanism as a result of decreases in the NGF protein (Fig. 4). The enhancement of the transcription of the NGF mRNA in the myocardium by the blockade of the spinal nerves may result from decreased NGF protein expression (Fig. 4) at the time of acute myocardial infarction. The results also indicate that the anaesthetic effect of ropivacaine at the concentration used in the current study was still effective within the time of the observation (the 180 min of observation after administration of the local anaesthetic) as reported previously.

We found only a slight reduction in systolic and diastolic arterial pressures after the blockade of spinal nerves, thoracotomy, and CAO, which may indicate that the attenuation of the NGF in the first 60 min after myocardial infarction was unlikely to be due to changes in haemodynamics. The inhibition of the NGF by the blockade of spinal nerves was also unlikely to result from the inhibition of expression of genes encoding NGF in the myocardium, because NGF mRNA expression was not reduced by spinal nerve blockade. In fact, mRNA was increased in the AMI animals with spinal nerve blockade 360 min after CAO (Fig. 5), indicating the enhancement of NGF mRNA transcription in the myocardium.
Taken together, we conclude that spinal nerves innervating the heart may play an important role in the up-regulation of the NGF in the myocardium early after acute myocardial infarction and can be inhibited by the blockade of spinal nerves using a local anaesthetic. These results may be relevant for patients with heart diseases or after coronary artery procedures.

**Supplementary material**

Supplementary material is available at *British Journal of Anaesthesia* online.

**Declaration of interest**

None declared.

**Funding**

This work has been supported by grants from the National Natural Science Foundation of China.

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