Cardiac cholinergic NO-cGMP signaling following acute myocardial infarction and nNOS gene transfer

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Dawson TA, Li D, Woodward T, Barber Z, Wang L, Paterson DJ. Cardiac cholinergic NO-cGMP signaling following acute myocardial infarction and nNOS gene transfer. Am J Physiol Heart Circ Physiol 295: H990–H998, 2008. First published July 11, 2008; doi:10.1152/ajpheart.00492.2008.—Myocardial infarction (MI) is associated with oxidative stress, which may cause cardiac autonomic impairment. We tested the hypothesis that acute MI disrupts cardiac cholinergic signaling by impairing nitric oxide (NO)-cGMP modulation of acetylcholine (ACh) release and whether the restoration of this pathway following cardiac neuronal NO synthase (nNOS) gene transfer had any bearing on the neural phenotype. Guinea pigs underwent four ligature coronary artery surgery (n = 50) under general anesthesia to induce MI or sham surgery (n = 32). In a separate group, at the time of MI surgery, adenovirus encoding nNOS (n = 29) or enhanced green fluorescent protein (eGFP; n = 30) was injected directly into the right atria, where the postganglionic cholinergic neurons reside. In vitro-evoked right atrial [3H]ACh release, right atrial NOS activity, and cGMP levels were measured at 3 days. Post-MI 24% of guinea pigs died compared with 9% in the sham-operated group. Evoked right atrial [3H]ACh release was significantly (P < 0.05) decreased in the MI group as was NOS activity and cGMP levels. Tetrahydrobiopterin levels were not significantly different between the sham and MI groups. Infarct sizes between gene-transferred groups were not significantly different. The nNOS-transduced group had significantly increased right atrial [3H]ACh release, right atrial NOS activity, cGMP levels, and decreased cAMP levels. Fourteen percent of the nNOS-transduced animals died compared with 31% mortality in the MI + eGFP group at 3 days. In conclusion, cardiac nNOS gene transfer partially restores the defective NO-cGMP cholinergic pathway post-MI, which was associated with a trend of improved survival at 3 days.

autonomic nervous system; gene therapy; sudden death; neuronal nitric oxide synthase; nitric oxide; guanosine 3′,5′-cyclic monophosphate

Myocardial infarction (MI) is associated with increased oxidative stress (40, 50), which may disrupt nitric oxide (NO) cyclic nucleotide signaling. This process has been implicated in impaired autonomic control in a number of chronic cardiovascular diseases (25, 32, 37, 43). In the peripheral nervous system under normal conditions, neuronal NO synthase (nNOS) facilitates the release of cardiac acetylcholine (ACh) (15, 26, 42), inhibits the release of cardiac norepinephrine (47), and decreases heart rate (10) by the modulation of intracellular calcium handling (53). Postsynaptically, nNOS is positioned in discrete subcellular domains where it assists in the regulation of sarcoplasmic reticulum calcium handling (49), excitation-contraction coupling (48), and β-adrenergic and muscarinic regulation of the L-type calcium channel current (ICaL) (16, 22, 24) in cardiac pacemaking.

In the spontaneously hypertensive rat (SHR), both NOS activity and cGMP levels are decreased in the right atria, where cholinergic intracardiac ganglia predominately reside (24, 25). This is correlated with impaired cardiac vagal vagal transmission, which can be restored by targeted nNOS overexpression (25). In humans (20, 38, 44) and dogs (46), the parasympathetic control of the myocardium is diminished in the early phase of MI. However, in the rat, post-MI, there is a compensatory increase in cardiac vagal tone coupled with a rise in right atrial nNOS mRNA and expression (51).

In the acute phase after MI, a period associated with increased vulnerability to arrhythmia and death, we tested the hypothesis that there would be an impairment of cardiac cholinergic NO-cGMP signaling. Furthermore, we also hypothesized that gene transfer of nNOS into the right atria would restore parasympathetic control of cardiac excitability since enhanced cardiac vagal activity is antiarrhythmic (17, 21, 52).

MATERIALS AND METHODS

Experiments were performed in accordance with the United Kingdom Animal Procedures Act of 1986 PPL 30/2130 and approved by the Institutional Animal Care and Use Committee. Furthermore, the investigation conformed with the American Physiological Society “Guiding Principles in the Care and Use of Animals.” All reagents were sourced from Sigma unless otherwise stated.

Animal model. The guinea pig has a well-characterized autonomic neural phenotype (27, 41) and has been successfully used to model ventricular fibrillation (18). However, guinea pigs have considerable cardiac arterial collateralization (39, 45), and evidence has been presented that the guinea pig is impossible to infarct with a single coronary artery ligation (39), although others report variable success with this approach (30). For this reason, the arterial supply of the anterior left ventricle was ligated in four separate locations.

All tissue was phenotyped at 3 days post-MI surgery. The first week post-MI is the vulnerable clinical window for fatal arrhythmias. At 72 h, Takimoto and colleagues (51) observed significant increases in nNOS expression in the atria and changes in autonomic function. At 7 days, these responses had diminished considerably (51). Three days is also the point at which, after MI in the rat, acute inflammatory changes start settling down and proliferation commences (19).

Surgery and anesthesia. Adult guinea pigs (Cavia Porcellus; body weight, 447 ± 8 g; n = 141; 32 sham-operated and 109 MI surgery) were induced under 4% isoflurane (Animal Care) for 30 min. Anesthesia was maintained using a mixture of oxygen and 2% to 3% isoflurane. IM buprenorphine was administered (0.15 μg/g; Alstoe Animal Health), and the animals were placed on a heated operating table for maintaining temperature control. They were monitored with a two-lead electrocardiogram, intubated, and ventilated. A left-sided thoracotomy was performed at the level of the fourth and fifth ribs anterolaterally. The pleural membrane was ruptured, and retractors were placed. The chest was re-expanded, and the pleural retractor and chest tube were removed. The thoracic cavity was sutured, and chest tube was removed. The thoracic cavity was sutured, and chest tube was removed.

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were used to expose the heart. A pericardectomy was performed. The left anterior descending coronary artery (LAD) and visible collaterals were tied off in four places. Specifically, the LAD was tied off at its origin and branch point. The circumflex artery was tied off at its origin, and the most prominent feeder from the septum was tied off as it entered the left ventricle. If the circumflex was not visible, as was common in the later guinea pigs infarcted, a deep ligature was then placed just above the LAD origin. Once the thoracotomy was repaired, isoflurane was discontinued. Subcutaneous isotonic dextrose-saline (20 μl/g; Baxter Healthcare) was administered at the end to ensure optimal hydration postsurgery. Animals were placed under intensive care monitoring for 18 h before being returned to holding facilities. Sham-operated animals underwent identical procedures with the exception of coronary artery ligation.

In vitro preparation. Three days after surgery, the guinea pigs were euthanized by cervical dislocation. A midline thoracotomy was performed, and the heart and mediastinum were removed and placed in guinea pig Tyrode solution that was continuously bubbled with 95% O2-5% CO2 and contained (in mmol/l) 120 NaCl, 4 KCl, 2 MgCl2, 0.1 NaH2PO4, 23 NaHCO3, 11 glucose, and 2 CaCl2 (pH 7.4) as previously described (28). The right and left atria and the ventricles were removed and used for either in vitro studies, prepared for histology, or snap frozen in liquid nitrogen.

Quantification of MI. Because the surgical model used required permanent ligation and the study ended at 3 days, there was a limit to histological methods that could be used to quantify infarct size. At 3 days, the triphenyltetrazolium-chloride (TTC) method would have ideally been used. The tissue could not, however, be reperfused due to the permanent ligation of the coronary arteries. Because of the time delay involved in harvesting the atria for in vitro analysis, slicing the ventricles and incubating them in TTC would have also significantly and unreliably overestimated infarct size due to further necrosis.

Although collagen formation does not peak until 2 wk after MI, it was decided to use the Picric acid-Sirius red (PSR) method, which has been widely used and well defined (11). For the purpose of this study, PSR staining was used as a relative quantitative marker of infarct size; that is, it underestimated real infarct size but allowed for intergroup comparisons since all animals were phenotyped at 3 days.

In brief, after isolation the ventricles were blotted dry. They were weighed, and the free wall of the right ventricle was removed. The septum and free wall of the left ventricle were then fixed into paraffin blocks, sliced into 8-μM sections, and stained with PSR as previously described (11). Every tenth section was analyzed by automated digital microscopy, and the results were collated and expressed as percentages of stained tissue over total tissue analyzed. As a further determinant of infarct size, the cardiac-to-body mass ratio was analyzed.

Immunohistochemistry. Immunohistochemistry was performed as previously described (14) with the following modifications. Right atria and ventricles were harvested and placed in 10% Formalin (4% formaldehyde solution) overnight for fixing. The tissue was then

**Fig. 1.** i: bright light microscopy image (20×) of cholinergic ganglia residing within the right atria wall. ii: representative slide showing Sirius red collagen staining of sham-operated guinea pig left ventricle. iii: Sirius red staining of myocardial infarction (MI) model guinea pig left ventricle. iv: significant difference between sham (n = 6) and MI (n = 6) left ventricle free wall collagen staining (**P < 0.001; unpaired t-test).**
transferred into a 30% sucrose phosphate-buffered saline solution for a further 24 h. Samples were then frozen down, and 40-μM transverse sections were placed in phosphate-buffered saline. To assess enhanced green fluorescent protein (eGFP); cholinergic colocalization, goat anti-choline acetyltransferase antibody (Chemicon) was used as a primary antibody with biotinylated horse anti-goat IgG (Vector) used as a secondary antibody, which was then further labeled with streptavidin Texas red. To assess nNOS; cholinergic colocalization, the preparation was prepped as for eGFP and then blocked using the streptavidin kit. Following this, the tissue was incubated in anti-nNOS polyclonal rabbit antibody (Zymed) with biotinylated goat anti-rabbit antibody (Vector) being used as the secondary. This was then labeled with streptavidin fluorescein. Tissue was then scanned and digitally photographed at ×20 magnification using a Nikon Eclipse TE2000-U inversion fluorescent microscope and appropriate filters.

**Measurement of nNOS protein expression and NOS activity.** Western blotting for nNOS in right atria was performed using standard techniques as described previously (42), using commercially available antibodies to nNOS (Zymed) and the Western lightening detection system (Pierce Elmore Life Sciences). Protein levels were expressed as a ratio of the optical densities (RD) of the nNOS bands relative to the control. Recombinant nNOS was used as the control. Samples were randomized, and the investigator was blinded until all quantitative analyses had taken place. Protein (32 μg) was loaded into each lane. Protein loading was controlled using the MemCode Reversible Protein Stain kit (Pierce). NOS activity in atria was quantified by measuring the conversion of [3H]-l-arginine (Amersham) to [3H]-l-citrulline using a procedure as previously described (25). The putatively specific endothelial NOS inhibitor [1-NAME (1-iminoethanol)] ornithine, dihydrochloride; Calbiochem] was added to the assay buffer at a concentration of 10 μg/assay. Radioactivity was quantified by liquid scintillation counting. The results are expressed in femtomoles citrulline per milligram protein per minute.

**Measurement of tissue cyclic nucleotides.** After atria were frozen in liquid nitrogen, cAMP and cGMP levels were determined as previously described (24). Tissues were minced in 800 μl ice-cold trichlo-roacetic acid (6%) and homogenized at 4°C. The homogenates were centrifuged at 2,000 g for 10 min at 4°C, and the supernatant was extracted with water-saturated ether three times and then dried using a SpeedVac concentrator (Savant). The dried samples were resuspended. 125I-cAMP or 125I-cGMP Biotrak assay system (Amersham) was used to measure the amount of the cAMP or cGMP level, respectively, radioimmunoassay using Amerlex-M, magnetic separation (Amersham). The pellet was treated with 500 μl NaOH (1N) and used for protein quantification.

**Tissue biotinperin levels.** Tetrahydrodriopterin (BH4) and total biotinperin were measured by high-performance liquid chromatography (HPLC) as previously described by Cai et al. (8). 

**[3H]ACh release.** Evoked [3H]ACh release was measured as previously described (25) with the following modifications. At 16 and 52 min, the atrium was stimulated for 1 min at 10 Hz. In 18 experiments, the specific nNOS inhibitor 4S-NAME (4-aminophenol)[aminooethyl][aminopentyl]-N'-nitroguanidine ([NNNG]; 5 μmol/l) was added to the solution from 30 min until the end of the experiment.

**Mortality analysis.** If animals were euthanized before reaching the 72 h end point of the study, they were categorized based on operation notes and autopsy findings as death from either obvious cause (for example, cardiac rupture) or unexplained (presumed sudden cardiac death). Blinded analysis was undertaken by one coauthor and another nonauthor. For the animals that were transduced with either nNOS or eGFP, the analysis of the effect of gene transfer on mortality was taken from 6 h post-surgery and transduction on the basis that prior work on the time course of cytomegalovirus (CMV) adenovirus expression has demonstrated that functional gene expression from nNOS or eGFP transduction can be detected at 6 h (23).

**Gene transfer.** At the time of surgery, post-coronary artery ligation, 5 × 10^10 virus particles of adenoviral vector with CMV promoter encoding nNOS (n = 29), or GFP as the control (n = 30), in 300 μl phosphate-buffered saline was directly injected into the right atrial wall and fat pad with direct visualization. The right atrial wall of the guinea pig has a rich cholinergic innervation (Fig. 1). The efficient transduction of the right atrial cholinergic neurons in the guinea pig using the above viral construct and injection via a blind subcutaneous approach has been previously demonstrated (42).

**Analysis, data presentation, and statistics.** Investigators were blinded to the samples they were analyzing. All data are presented as means ± SE. SPSS 15.0 was used for statistical analyses. Data sets were tested for normality, and if they were skewed and/or failed to fit a normal distribution using both the Kolmogorov-Smirnov and Shapiro-Wilk tests (P < 0.2), then nonparametric analysis was used. For comparing two means of a parametric data set, t-tests or two-factor ANOVA tests were then used. For the analysis of nonparametric data sets, the Mann-Whitney’s U test was used for independent samples, whereas the Wilcoxon’s signed ranks test was used for paired data. Kaplan-Meier tests were used for mortality unless the assumptions (based on χ^2) were not met, in which case the more robust Fishers’ exact test was used. Significance was defined as P < 0.05.

**RESULTS**

At 3 days postsurgery, the MI group had a significantly higher percentage of collagen staining of the left ventricle and septum than the sham group (Fig. 1iv). The staining method was further validated by a microscopic examination of the red myofilaments, which showed morphological changes typical of infarction (Fig. 1iii). The MI group also had a significantly higher percentage of ventricle to body mass at time of eutha-
nasia compared with that of the sham group (sham, 0.026 ± 0.01%, n = 36 vs. infarct, 0.029 ± 0.01%, n = 43; P < 0.05).

Molecular phenotype. There was a trend of decreased expression of nNOS protein in the right atria MI compared with the sham group, although this failed to reach significance (sham, 0.89 ± 0.03 RD, n = 6 vs. 0.65 ± 0.17 RD, n = 6, P = 0.11). However, at 3 days post-MI, there was reduced atrial NOS activity compared with that of sham-operated animals (Fig. 2i). cGMP levels were also significantly reduced in the MI compared with the sham group (Fig. 2ii). There was no significant difference in cAMP levels (sham, 106.9 ± 8.9 pmol/mg, n = 10 vs. MI, 131.9 ± 11.3 pmol/mg, n = 10) between groups. Right atrial BH4 levels (sham, 14.1 ± 2.1 pmol/mg, n = 9 vs. MI, 18.8 ± 2.3 pmol/mg, n = 8) were not decreased in the MI group, indicating that molecular coupling of nNOS was preserved.

In vitro ACh release. To establish the stability of the preparations, a time control of evoked right atrial [3H]ACh release in six guinea pigs was performed, showing no decline in percentage release over 105 min (Fig. 3i). Three days post-MI, there was a significant decrease in in vitro-evoked right atrial [3H]ACh release in the MI group compared with the sham animals (Fig. 3iv). Both groups had significantly decreased evoked [3H]ACh release after incubation with the nNOS inhibitor NNNG (Fig. 3iv), suggesting that the NO-cGMP pathway was still viable, albeit significantly impaired post-MI.

Effect of nNOS gene transfer. Infarct sizes were not significantly different between groups (MI + nNOS, 6.5 ± 0.8%, n = 11 vs. MI + eGFP, 7.3 ± 0.7%, n = 8). There was no significant difference between the percentage of ventricle mass and body weight at time of terminal procedure between groups (eGFP, 0.029 ± 0.01%, n = 22 vs. nNOS, 0.029 ± 0.01%, n = 20).

Immunohistochemistry. Two guinea pigs were used for the validation of efficacy of gene transfer; one was transduced with eGFP at the time of MI surgery, and the other with nNOS. eGFP predominately colocalized in endothelial cells although it was also expressed in cholinergic neurons. No eGFP was detected in cardiac myocytes or in the right ventricle. nNOS was detected in small amounts in the control atria, whereas in the nNOS-treated group, there was significant transduction (Fig. 4).
Effect of gene transfer on in vitro \( \text{ACh} \) release. The nNOS transduced group had increased right atrial \( [3\text{H}]\text{ACh} \) release compared with that of the eGFP-treated group. Both groups had decreased evoked \( [3\text{H}]\text{ACh} \) release after incubation with the nNOS inhibitor NNNG (Fig. 6).

Mortality. Nine percent (3/32) of the sham animals died at 3 days as opposed to 24% (12/50) of the infarct group (Fig. 7i). One of the sham animals died from complications due to wound infection, whereas three from the MI group had a hemothorax, one of these being secondary to ventricular rupture.

At 3 days, 30% (9/30) from the eGFP + MI group died from all-cause death, whereas 14% (4/29) died in the nNOS + MI group (Fig. 7ii). After 6 h, no animals died from unexplained causes in the nNOS group compared with 20% of the eGFP transduced guinea pigs (Fig. 7iii).

DISCUSSION

The new findings presented here are: 1) at 3 days post-MI, there is disruption of the NO-cGMP pathway that is correlated with impaired evoked atrial ACh release; and 2) nNOS gene transfer into cholinergic neurons partially restores cardiac parasympathetic function, and this is associated with a trend suggesting improved survival at 3 days post-MI.

Cardiac vagal phenotype and gene transfer. Clinically MI may induce the disruption of autonomic balance as evidenced by decreased heart rate variability (3, 38), baroreflex sensitivity (44), and other markers of vagal control (20). Furthermore, surgically induced MI decreases vagal activity in dogs (46) although the converse has been shown in rats (51). Takimoto and colleagues (51) found that there was increased right atrial nNOS protein expression at 3 days after MI that was also associated with enhanced vagal responsiveness in vivo compared with that of sham animals. The current study found opposite results at both the molecular and functional level when ACh release was directly measured. These conflicting results may be partially explained by differing assessments of vagal function, but this would not explain the disparity in the nNOS measurements. nNOS is a ubiquitous protein that is expressed across species. It is unlikely that a difference in species would simply explain the difference in expression as a result of similarly induced MI. Inflammatory cytokines, which are known to regulate protein expression, are released from the surgical sites and may effect tissues in close proximity. Furthermore, the oxidative load induced post-MI may scavenge NO or reduce nNOS activity. It appears that there is a complex interplay among inflammatory, oxidative, and nitrosative elements contributing to the observed disparate outcomes. The differing spatial distance between the right atrium and the surgical site could conceivably explain the differences in response. In dogs and humans, the site of infarct is relatively distant to the right atrium. Overall, the autonomic results from the guinea pig mimic closely the responses observed in humans and dogs.

Parasympathetic impairment is a powerful independent negatively prognostic predictor of arrhythmia and death (2, 12, 13, 34); therefore, various strategies have been used to enhance cardiac vagal function. Direct electrical stimulation of the vagus improves survival in rats with heart failure (36). In exercising dogs with a healed MI, vagal stimulation was able to...
prevent ventricular fibrillation during induced myocardial ischemia (52). Exercise training has been shown to confer protection post-MI through improving both parasympathetic and sympathetic indexes of autonomic function in dogs (4-7) and humans (33). Furthermore, it has been demonstrated in mice that exercise induces an increase in right atrial nNOS expression resulting in increased vagal responsiveness (14, 15). This autonomic phenotype is absent in nNOS mutant mice and can be restored by right atrial nNOS gene transfer (14). Moreover, directly targeting nNOS into the cardiac vagus in the anesthetized pig increases baroreflex sensitivity and heart rate response to vagal nerve stimulation (23).

In the current study, nNOS gene transfer into the right atria was a successful strategy to improve vagal function and restore autonomic balance in the acute phase post-MI. This is consistent with recent findings that reported that cholinergic gene transfer of nNOS in the SHR rescued the impaired vagal phenotype (25).

The CMV promoter is efficacious for transducing atrial cholinergic neurones (25, 42). However, this promoter is promiscuous and will drive expression in other cell types. For instance, adenoviral gene transfer of CMV.nNOS into the right atrium of the SHR also expresses in sympathetic neurones and cardiac myocytes, resulting in decreased sensitivity to norepinephrine via increased cGMP-dependent inhibition of I_{Ca,L} in the sinoatrial node (24). Neuronal NOS gene transfer may have had additional beneficial effects on the outcome by decreasing cardiac sympathetic activity (53, 54) and β-adrenergic activation of I_{Ca,L} (24) as gene transfer decreased cAMP levels.

Fig. 5. i: embedded bitmap of Western blot for nNOS showing standard (STD), eGFP (E), and nNOS (N) transduced right atria. ii: Western blot analysis showing increased nNOS protein in the Ad.nNOS- compared with the Ad.eGFP-transduced MI guinea pigs (n = 6 in each group; **P < 0.01, unpaired Mann-Whitney’s U exact test). iii: right atrial NOS activity was increased in the MI + nNOS guinea pigs compared with the MI + eGFP group (MI + eGFP, n = 11 vs. MI + nNOS, n = 11; ††P < 0.01; unpaired Mann-Whitney’s U exact test). iv: cGMP levels were increased in the right atria of MI + nNOS compared with MI + eGFP guinea pigs (MI + eGFP, n = 15 vs. MI + nNOS, n = 13; **P < 0.01; unpaired Mann-Whitney’s U exact test). v: right atrial cAMP levels were lower in the nNOS- compared with the eGFP-transduced group (MI + eGFP, n = 15 vs. MI + nNOS, n = 13; *P < 0.05; 1-tailed, independent t-test for unequal variances). MM, molecular mass.
Therefore, the trend of improved mortality is not necessarily solely due to increased nNOS expression in cholinergic neurons since nNOS is probably upregulated in adjacent cells, inhibiting sympathetic neurotransmission and β-adrenergic

Fig. 6. (i): representative raw data plot of right atrial \(^3\)H ACh release with epicardial stimulation pre- (S1) and post- (S2) addition of nNOS inhibitor NNNG in MI + eGFP guinea pig. (ii): representative raw data plot of right atrial \(^3\)H ACh release with epicardial stimulation pre- (S1) and post- (S2) addition of NNNG in MI + nNOS guinea pig. (iii): nNOS gene transfer significantly increased right atrial \(^3\)H ACh release with epicardial stimulation at 3 days post-MI (n = 6) compared with that of eGFP (n = 6)-transduced animals (*\(P < 0.05\); unpaired Mann-Whitney’s U exact test). Inhibition of nNOS caused a significant decrease in \(^3\)H ACh release in the MI + eGFP and MI + nNOS groups (**\(P < 0.05\); Wilcoxon’s signed ranks exact test). AMI, acute MI.

Fig. 7. (i): survival graph for guinea pigs post-sham (n = 32) or infarct (n = 50) surgery showing more deaths in the MI group up to experimental endpoint at 72 h. There was significantly more mortality in the MI group (*\(P < 0.05\); Kaplan-Meier Log-Rank (Mantel Cox)). Survival graphs, from either all-cause (ii) or unexplained death (iii), of guinea pigs post-infarct surgery with Ad.eGFP (n = 30) or Ad.nNOS (n = 29) transduction up to experimental endpoint at 72 h. There was significantly improved survival from 6 h (dotted line), when viral transduction of proteins encoded with the CMV promoter have first been shown to express in vivo, to end point in the nNOS compared with the eGFP group in both the all-cause (**\(P < 0.05\); Kaplan-Meier Log-Rank (Mantel Cox)) and unexplained (*\(P < 0.05\); Fisher’s exact test) death groups.
signaling, which would have a further beneficial effect on sympathovagal balance.

**Limitations.** Due to the lack of telemetry data, it was only possible to broadly classify the deaths as being due to explained or unexplained causes. It is significant that simply by altering the peripheral cardiac vagal capacity for ACh release by nNOS amplification, there was improved mortality from both all-cause and unexplained death post-major MI. However, this study was not designed to test the hypothesis that nNOS gene transfer improves cardiac outcome post-MI. To do this systematically, animals would need to be telemetered with increased numbers to enhance the statistical power. For instance, designing the study to look for a 50% improvement in mortality would require 526 guinea pigs for 0.05% significance and 90% power.

It is possible that the gene transfer shifted nNOS to a higher baseline and that there were similar reductions in cholinergic regulation, which were obscured by the effect of the amplification at the time of surgery. If sham animals had been transduced with nNOS, this potential effect could have been investigated; that is, if both groups had similar profiles post-gene transfer it could be clearly stated that gene transfer was restorative, whereas if sham transduced animals had an increase in the NO-cGMP signaling pathway, then the effect of gene transfer could be said to be increasing gain of function if the MI reduced. This, however, remains a point of speculation. Nevertheless, nNOS gene transfer improved function compared with that of the eGFP-transduced animals. This study targeted one limb of the cardiac neural axis and showed MI can disturb post-ganglionic circuits at the end organ level. Clearly, we do not rule out that MI itself may also interfere with the central integration of reflex information coming from the heart.

**Perspectives**

Diminished cardiac parasympathetic activity is a negative prognostic indicator for sudden cardiac death, especially after MI (1, 31, 35), whereas the upregulation of cardiac parasympathetic activity, as seen in response to exercise training, is a positive prognostic indicator (33). Recent evidence suggests that a reduction in nNOS-derived NO may contribute to dysfunctional neural control of the myocardium that is associated with several cardiovascular diseases (9, 25, 29, 53–56). We have demonstrated that that the upregulation of nNOS utilizing a viral gene transfer approach may restore the NO-cGMP facilitation of ACh release in the acute phase post-MI. Further understanding the role of nNOS in the cardiacmyocyte and cardiac neural network by selectively targeting tissues may lead to the development of novel therapeutic strategies.

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