Efferent Vagal Nerve Stimulation Protects Heart Against Ischemia-Induced Arrhythmias by Preserving Connexin43 Protein

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Background—Myocardial ischemia (MI) leads to derangements in cellular electrical stability and the generation of lethal arrhythmias. Vagal nerve stimulation has been postulated to contribute to the antifibrillatory effect. Here, we suggest a novel mechanism for the antiarrhythmogenic properties of vagal stimulation during acute MI.

Methods and Results—Under anesthesia, Wistar rats underwent 30 minutes of left coronary artery (LCA) ligation with vagal stimulation (MI-VS group, n=11) and with sham stimulation (MI-SS group, n=12). Eight of the 12 rats in the MI-SS group had ventricular tachyarrhythmia (VT) during 30-minute LCA ligation; on the other hand, VT occurred in only 1 of the 11 rats in the MI-VS group (67% versus 9%, respectively). Atropine administration abolished the antiarrhythmogenic effect of vagal stimulation. Immunoblotting revealed that the MI-SS group showed a marked reduction in the amount of phosphorylated connexin43 (Cx43), whereas the MI-VS group showed only a slight reduction compared with the sham operation and sham stimulation group (37±20% versus 79±18%). Immunohistochemistry confirmed that the MI-induced loss of Cx43 from intercellular junctions was prevented by vagal stimulation. In addition, studies with rat primary-cultured cardiomyocytes demonstrated that acetylcholine effectively prevented the hypoxia-induced loss of phosphorylated Cx43 and ameliorated the loss of cell-to-cell communication as determined by Lucifer Yellow dye transfer assay, which supports the in vivo results.

Conclusions—Vagal nerve stimulation exerts antiarrhythmogenic effects accompanied by prevention of the loss of phosphorylated Cx43 during acute MI and thus plays a critical role in improving ischemia-induced electrical instability. (Circulation. 2005;112:164-170.)

Key Words: arrhythmia • connexins • electrical stimulation • gap junctions • vagus nerve

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cute myocardial ischemia results in a dramatic reduction of tissue pH, an increase in interstitial potassium levels, an increase in intracellular calcium concentration, and neurohumoral changes, all of which contribute to the development of electrical instability that leads to life-threatening cardiac arrhythmias.1,2 In particular, cell-to-cell electrical uncoupling of ventricular myocytes plays an important role in arrhythmogenesis during acute and chronic ischemic heart disease.3–6

Gap-junction channels, composed of highly homologous proteins known as connexins in vertebrate species, have been implicated in the electrical coupling of excitable tissues, such as cardiac muscles. There are essentially 2 subtypes of connexins (Cx), Cx40 and Cx43, in the adult heart muscle. Cx43 is predominantly expressed in ventricular tissue, whereas Cx40 is mainly found in atrial tissue and in the conduction system.7 The protein content of ventricular Cx43 is remarkably reduced in ischemia8,9 and heart failure.10,11

Gene-targeting studies demonstrate that reduced expression of Cx43 increases the incidence of ventricular tachyarrhythmias12 and causes a significant reduction in conduction velocity in mice during acute myocardial ischemia.13 These results suggest that the dysfunction of Cx43 in cardiomyocytes could be one of the components of the substrate that promotes lethal ventricular tachyarrhythmias.

With regard to life-threatening arrhythmias in acute ischemia, the effect of vagal nerve stimulation (VS) has been reported to prevent ventricular fibrillation in dogs.14 Recently, VS therapy markedly improved long-term survival in an animal model of chronic heart failure after myocardial infarction,15 and earlier studies have demonstrated that ventricular arrhythmia is one of the major causes of death in chronic heart failure conditions.16 However, the mechanisms of VS on myocardial infarction remain unknown. With these in mind, we hypothesized that VS would exert an antiarrhythmogenic effect even in acute myocardial ischemia by target-
ing the gap junctions. Therefore, in the present study, using both in vivo acute ischemia model in rats and in vitro primary cultured cardiomyocytes from neonatal rats, we examined the effects of VS on arrhythmogenesis during acute myocardial ischemia.

Methods

The care and use of animals were in strict accordance with the guiding principles of the Physiological Society of Japan.

In Vivo Arrhythmia Study

Male Wistar rats (SLC, Japan) weighing 270 to 300 g were assigned to 6 groups receiving the following treatments: sham-operated rats treated with sham stimulation (SO-SS, n = 6), sham-operated rats treated with vagal stimulation (SO-VS, n = 5), myocardial ischemia rats with sham stimulation (MI-SS, n = 12), myocardial ischemia rats with vagal stimulation (MI-VS, n = 11), myocardial ischemia rats with both vagal stimulation and atropine administration (MI-VS-Atrop, n = 6), and myocardial ischemia rats preconditioned by vagal stimulation (pcVS-MI, n = 9).

Acute Ischemia Model

After induction of anesthesia, the rat was ventilated artificially with a volume-controlled rodent respirator (model 683, Harvard Apparatus) at 80 strokes per minute. Anesthesia was maintained through the use of 1.2% halothane during surgical procedures and 0.6% halothane during data recording. Left ventricular MI was induced by 30 minutes of left coronary artery (LCA) ligation. In sham-operated rats, we loosely tied a suture around the LCA without arterial occlusion. For measurement of arterial pressure, a polyethylene tubing (PE-10, Becton Dickinson) that was filled with saline and connected to a fluid-filled transducer (DX-300, Viggo-Spectramed) was cannulated into the right femoral artery. Experimental solutions were infused through another polyethylene tubing, which was cannulated into the right femoral vein. Area measurements were performed with Image-Pro version 4.0 (Media Cybernetics). Evans blue dye was injected via the femoral vein at the end of 30 minutes of left coronary artery occlusion. For immunoblotting, proteins separated by SDS-PAGE with 15% polyacrylamide gels were transferred electrophoretically to polyvinylidene difluoride sheets (Millipore). After 1 hour of blocking in the 4% skimmed milk solution, the membrane was incubated overnight with anti-Cx43 antibody (71-0700, Zymed) diluted 1:100. Biots were then incubated for 1 hour with horseradish peroxidase–conjugated goat anti-rabbit IgG, developed with an ECL chemiluminescence reagent (Amersham), and exposed to a medical x-ray film. Equal protein content in all the samples was confirmed by Coomassie brilliant blue staining.

Immunohistochemistry and Confocal Microscopy

Transmural blocks of left ventricular myocardium from selected hearts were immersed in a fixative containing 4% paraformaldehyde and 0.1 mol/L phosphate buffer (pH 7.4), embedded in paraffin, and sectioned at a thickness of 4 μm. Sections were deparaffinized, placed in citrate buffer, and boiled in a microwave oven for 10 minutes to enhance specific immunostaining. The sections were then incubated overnight with anti-Cx43 antibody diluted 1:100 and then incubated for 2 hours in Alexa546-conjugated goat anti-rabbit IgG (Molecular Probes) diluted 1:100. Fluorescence of Alexa546 was observed with a confocal laser scanning microscope system (FV300, Olympus). Reconstructed projection images were obtained from serial optical sections recorded at an interval of 0.5 μm.

Primary Culture Study

We used neonatal rat primary cultured cardiomyocytes to investigate the direct action of acetylcholine (ACH), a neurotransmitter released by VS on myocardial cells. We conducted this protocol separately from the in vivo arrhythmia study because the experimental preparation of Evans blue dye injection for risk area assessment did not allow us to perform immunoblotting and immunohistochemical assay of excised hearts. For each group, we prepared and analyzed 5 hearts.

Dye Transfer Assay

To assess the effect of ACh on cell-to-cell communication, we monitored dye transfer in primary cultured cardiomyocytes with Lucifer Yellow CH (LY; lithium salt, Molecular Probes). Cells in the culture medium were viewed on a Zeiss microscope equipped with fluorescence illumination and FITC filters. The emitted fluorescent light was projected onto a CCD camera system (C4742-98-24ER, Ando et al Vagal Stimulation and Connexin43

Risk Area Assessment

To confirm the ischemic area induced by LCA ligation, 2 mL of 2% Evans blue dye was injected via the femoral vein at the end of 30 minutes of coronary artery ligation. The area at risk was determined by negative staining with Evans blue. Area measurements were determined with Image-Pro version 4.0 (Media Cybernetics).
Hamamatsu Photonics). LY was injected electrophoretically via a conventional intracellular microelectrode made from borosilicate glass capillaries. The outer diameter of microelectrodes was 0.15 to 0.25 μm, and the tip resistance of these electrodes filled with 3% LY was 0.6 to 1.4 GΩ. An inward square-pulse current (20 to 40 nA) of 0.5-second duration was injected at a frequency of 1 Hz for 2 to 3 minutes using the circuitry of an amplifier (707, WPI) and a voltage pulse generator (SEN7203, Nihon Kohden). For induction of the hypoxic condition, cardiomyocytes were treated for 5 hours with 0.2 mM CoCl2,19 because microinjection methods are not suitable for gas-tight chamber experiments with N2 substitution. Cells were treated either with ACh (0.5 mM/L) alone or with both ACh and atropine (0.1 mM/L) before chemical hypoxia. Dye injection was performed at room temperature (24°C to 26°C).

Statistical Analysis
Time of onset of the first run of VT was analyzed by the Kaplan-Meier method, and comparisons were made with the Mantel-Haenszel log-rank test. A nonparametric test for comparison of treatments with a control was performed by a Mann-Whitney U test with Bonferroni adjustment. Differences were considered significant at P<0.05. Values are expressed as mean±SD.

Results

In Vivo Arrhythmia Study

Effects of VS on Acute Ischemia-Induced VT
Figure 1 illustrates an example of the VT observed after LCA ligation with sham stimulation in the MI-SS group. As demonstrated in this case, VT followed by ventricular fibrillation was often detected after LCA ligation without VS. Figure 2 shows the time of onset and the incidence of the first spontaneous VT after LCA ligation. Eight of 12 rats in the MI-SS group experienced VT; on the other hand, only 1 of 11 in the MI-VS group developed VT during the 30-minute LCA ligation (67% versus 9%, P=0.005). VS achieved an 87% reduction in the relative incidence ratio of VT. In all cases, the first runs of VT were observed within 15 minutes after LCA ligation. Atropine administration abolished the anti-VT effects of VS; 4 of 6 rats developed VT (Figure 2). No VT was detected in either the SO-SS or SO-VS groups.

To exclude vagally induced bradycardiac effects on the incidence of VT, a group of 9 rats were subjected to preconditioning by VS (pcVS-MI group; see Methods). There was no significant difference in heart rate between the MI-SS and pcVS-MI groups immediately before and after LCA ligation (Table). Nevertheless, only 2 of 9 rats in the pcVS-MI group developed VT (22%, P=0.04 versus the MI-SS group).

Risk Area Assessment and Hemodynamic Parameters
There was no significant difference in risk area between the MI-SS and MI-VS groups (55±6% versus 59±3%). The differences in heart rate between groups with or without VS reached ≈50 bpm, and after 30-minute ischemia, only the MI-VS group had a significantly lower arterial pressure than the SO-SS group (Table).

Excised Heart Study

Effects of VS on Cx43 Expression
The polyclonal anti-Cx43 antibody in the present study showed closely spaced bands migrating between 43 and 46 kDa and a faint band migrating at 41 kDa. Previous reports20 showed closely spaced bands migrating between 43 and 46 kDa and a faint band migrating at 41 kDa. The polyclonal anti-Cx43 antibody in the present study showed closely spaced bands migrating between 43 and 46 kDa and a faint band migrating at 41 kDa. The polyclonal anti-Cx43 antibody in the present study showed closely spaced bands migrating between 43 and 46 kDa and a faint band migrating at 41 kDa. The polyclonal anti-Cx43 antibody in the present study showed closely spaced bands migrating between 43 and 46 kDa and a faint band migrating at 41 kDa.

Effects of VS on Cx43 Localization
To investigate the distribution of Cx43 during acute ischemia with or without VS, we performed confocal image analysis of left ventricular tissues stained with anti-Cx43 antibody. As shown in Figure 4A, localization of immunoreactive signals

![Figure 1](image1.png) Example of run of VT induced by LCA ligation in rat treated with sham stimulation. In this case, ventricular fibrillation was initiated after ventricular tachycardia. SAP indicates systemic arterial pressure.

![Figure 2](image2.png) Incidence and onset of first VT after LCA ligation. Myocardial ischemia rats treated with sham stimulation (MI-SS, ●, n=12) and with vagal stimulation (MI-VS, ■, n=11). VS significantly (P=0.005 vs MI-SS group) decreased incidence of ischemia-induced VT. In contrast, atropine administration blocked antiarrhythogenic effect by VS during acute MI (MI-VS-Atr, ▲, n=6). Preconditioning by VS significantly (P=0.04 vs MI-SS group) decreased incidence of ischemia-induced VT (pcVS-MI, ○, n=9).

![Figure 3A](image3A.png) Time of onset and the incidence of first runs of VT were analyzed by the Kaplan-Meier method, and comparisons were made with the Mantel-Haenszel log-rank test. A nonparametric test for comparison of treatments with a control was performed by a Mann-Whitney U test with Bonferroni adjustment. Differences were considered significant at P<0.05. Values are expressed as mean±SD.

![Figure 3B](image3B.png) Example of run of VT induced by LCA ligation in rat treated with sham stimulation. Ventricular fibrillation was detected after LCA ligation. Atropine administration abolished the anti-VT effects of VS; 4 of 6 rats developed VT (Figure 2). No VT was detected in either the SO-SS or SO-VS groups.

![Figure 4A](image4A.png) Incidence and onset of first VT after LCA ligation. Myocardial ischemia rats treated with sham stimulation (MI-SS, ●, n=12) and with vagal stimulation (MI-VS, ■, n=11). VS significantly (P=0.005 vs MI-SS group) decreased incidence of ischemia-induced VT. In contrast, atropine administration blocked antiarrhythogenic effect by VS during acute MI (MI-VS-Atr, ▲, n=6). Preconditioning by VS significantly (P=0.04 vs MI-SS group) decreased incidence of ischemia-induced VT (pcVS-MI, ○, n=9).

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in the SO-SS group was restricted to intercellular junctions, consistent with the gap junctions and intercalated disks. In contrast, the Cx43 signal was reduced dramatically in the MI-SS group (Figure 4B); however, the Cx43 signal in the MI-VS group was almost comparable to the level in the SO-SS group (Figure 4C). These observations indicate that the loss of phosphorylated Cx43 during acute ischemia was prevented by VS.

**Primary Culture Study**

**Effects of Hypoxia and ACh on Cx43 Expression**

To examine the muscarinic effect on hypoxia-induced loss of phosphorylated Cx43, immunoblot analysis was performed in primary cultured cardiomyocytes. Under normoxic conditions, addition of ACh for 30 minutes significantly increased the phosphorylated isoform of Cx43 compared with the control group (Figure 5). Although hypoxia for 30 minutes decreased phosphorylated Cx43, ACh treatment prevented this hypoxia-induced loss of Cx43. Atropine inhibited the preserving effects of ACh. Thus, these results indicate that ACh preserved phosphorylated Cx43 through a muscarinic receptor during 30-minute hypoxia.

**Effects of Hypoxia and ACh on Intercellular Coupling and Beating Rate**

To assess whether the induction of phosphorylated Cx43 by ACh results in a functional effect on cell-to-cell communication, LY dye transfer analysis was performed in primary cultured cardiomyocytes. As shown in Figure 6A, under normoxic conditions, LY injected into a cardiomyocyte diffused into other cardiomyocytes around the injected cell. In contrast, LY injected into a cardiomyocyte under chemical hypoxic conditions was confined to the injected cell, and no dye coupling with other cardiomyocytes was observed (Figure 6B); on the other hand, ACh administration preserved cell-to-cell communication (Figure 6C). Atropine treatment inhibited the preventive effect of ACh on hypoxia-induced uncoupling (Figure 6D). Under normoxic conditions, ACh treatment remarkably induced dye coupling between cardiomyocytes (Figure 6E).

Under normoxic conditions, the rate of spontaneous beating was $62\pm13$ bpm ($n=5$ experiments), and ACh did not slow the beating rate. Chemical hypoxia with CoCl$_2$ stopped the beating; however, even under hypoxic conditions, ACh preserved spontaneous beating. In the presence of atropine, ACh failed to prevent the hypoxia-induced cessation of beating. The results of Cx43 immunoblotting under chemical hypoxic conditions were identical to those under hypoxia with $<2\%$ O$_2$ (data not shown).

**Discussion**

In the present study, we investigated whether VS could attenuate acute MI-induced arrhythmogenic properties via modulation of a principal cardiac gap-junction protein, Cx43. Our results provide novel evidence that VS effectively...
inhibits loss of the phosphorylated isoform of Cx43 during acute MI. Although the precise mechanism by which VS modulates the dephosphorylation of Cx43 remains unknown, it is most likely that VS exerts its antiarrhythmogenic effects on ischemic ventricular myocytes through the preserved function of Cx43.

**VS and Antiarrhythmogenic Properties**

VS has already been reported to prevent ventricular fibrillation in dogs. In the present study, we hypothesized that VS exerts its antiarrhythmogenic properties by maintaining electrical coupling with ventricular cardiomyocytes as a result of prevention of Cx43 dephosphorylation induced by acute MI. However, because VS simultaneously evokes a bradycardiac effect, the question remains whether the heart rate deceleration caused by VS is a primary mechanism for antiarrhythmic properties during MI. In a preliminary study, we confirmed that short-term exposure of cultured cardiomyocytes to ACh only before hypoxia prevented the hypoxia-induced loss of phosphorylated Cx43 (unpublished observations, 2004). Therefore, it is conceivable that ACh had a cardioprotective effect independent of the heart rate–slowing mechanism during hypoxia or ischemia. To further clarify such a preconditioning effect in vivo experiments, we examined whether hearts preconditioned by VS were susceptible to ischemia-induced VT and whether pcVS prevented the ischemia-induced loss of Cx43. As expected, we confirmed that pcVS exerted its antiarrhythmogenic effects and sustained the level of phosphorylated Cx43 during ischemia. These results suggest that VS or ACh had a cardioprotective effect independent of the heart rate–slowing mechanism.

It is well recognized that Cx43, which is the principal component of ventricular gap-junction proteins, contributes to intercellular communication and electrical coupling. Beardslee et al. showed that Cx43 undergoes marked dephosphorylation during the process of electrical uncoupling induced by ischemia. Genetically engineered Cx43-deficient (Cx43^{-/-} or Cx43^{-/-/-}) mice have been reported to be markedly susceptible to ischemia-induced VT,12,13,22 In the present study, VS drastically reduced the incidence of VT and prevented the loss of phosphorylated Cx43 during acute MI. Therefore, functional preservation of Cx43 by VS would play an important role in antiarrhythmogenic properties during acute MI.

The result that ACh administration ameliorated the hypoxia-induced loss of dye coupling in cardiomyocytes is consistent with that of ACh-induced upregulation of phosphorylated Cx43. Under normoxic conditions, ACh did not slow down the spontaneous beating rate of cardiomyocytes. Hypoxia stopped the beating and diminished the phosphorylated isoform of Cx43; however, even under hypoxic conditions, ACh preserved the spontaneous beating and the phosphorylated isoform of Cx43. Therefore, it is conceivable that ACh has a cardioprotective effect independent of the beating rate.

Upregulation of Cx43 has been reported to accelerate spontaneous beating in cultured cardiomyocytes. Moreover,
cultured cardiomyocytes from genetically engineered Cx43-deficient (Cx43−/−/−) mice demonstrated slow spontaneous beating rates and were poorly synchronized with each other compared with wild-type cultured cardiomyocytes. From these findings and the present results, we speculate that ACh exerts its antiarrhythmogenic properties on ischemic or hypoxic hearts by preserving Cx43 and that such a beneficial effect is independent of its bradycardiac effect.

The spatial distribution of Cx43 can influence electrical stability of the heart. A more recent study by Poelzing and Rosenbaum has shown that Cx43 is a short-lived protein with a half-life of only 1 to 3 hours in adult hearts and that both lysosomal and proteasomal degradation play distinct roles in the life cycle of Cx43. Our observations in the present study suggest that VS may prevent the ischemia-induced loss of Cx43 as a consequence of inhibition of its degradation pathway. Third, VS is also postulated to suppress excessive inflammation. Recently, Tracey and colleagues identified a novel molecular link between the vagus nerve system and an antiinflammatory response to disease. They suggest that VS exerts an antiinflammatory effect via the nicotinic ACh receptor 7-subunit expressed in macrophages. The release of tumor necrosis factor- from macrophages is inhibited by nicotinic stimulation. In contrast, the present results indicate that VS exerts its antiarrhythmogenic effects via muscarinic cholinergic receptors.

Study Limitations
In the present study, we did not measure the myocardial interstitial level of ACh at the ischemic region during VS. Therefore, it is unclear whether the cardiac vagal efferent fiber innervating the ischemic region can release its neurotransmitter in response to electrical stimulation. An in vivo microdialysis technique has enabled monitoring of the local concentration of neurotransmitters such as catecholamines, amino acids, and ACh. In the cat heart, Kawada et al showed that VS increased the myocardial interstitial ACh level even in the ischemic region (e-mail, February 12, 2004). Although their previous study showed that acute MI induced the nerve-firing independent release of ACh from the vagal terminal, the electrical stimulation of the vagal efferent during acute MI produced the significant additive release of ACh to the myocardial interstitial space. Such an additional release of ACh in response to electrical stimulation during acute MI would play an important role in Cx43 preservation in the ischemic region.

Conclusion and Future Aspects
The present study demonstrates that VS exerts antiarrhythmogenic effects during acute MI accompanied by prevention of the loss of phosphorylated Cx43. The preserved function of Cx43 may improve electrical instability during acute MI. In view of the present results, we can provide an alternative therapeutic strategy with a neural interface approach. We have already developed the sympathetic interface approach for the treatment of central baroreflex failure in rats. To establish the therapeutic strategy shown here, further studies are required.

Acknowledgments
This study was supported by Health and Labor Sciences Research Grant (H15-PHYSI-001) for Advanced Medical Technology from...
the Ministry of Health, Labor and Welfare of Japan. We thank Ken-ichi Yagyu for his technical assistance.

References


CLINICAL PERSPECTIVE

Increased cardiac vagal tone reduces ventricular arrhythmias during acute myocardial ischemia and has been linked to a lower risk of sudden arrhythmic death. Although the benefit of bradycardia associated with vagal tone is well appreciated, this may not be the only benefit. Cardiomyocytes are electrically coupled to one another through gap junctions. This coupling is critical to maintenance of cardiac electrical stability. Uncoupling occurs during ischemia and promotes heterogeneity of repolarization and slowing of conduction, with proarrhythmic effects. In the present study, short-term vagal stimulation (VS) was applied before or during acute ischemia in rats. VS protected against ventricular arrhythmias. Furthermore, VS preserved a phosphorylated form of connexin 43 (Cx43), a major subtype of gap-junction proteins in ventricles. In vitro studies of rat primary-cultured cardiomyocytes showed that ACh, a vagal efferent neurotransmitter, effectively prevented hypoxia-induced loss of phosphorylated Cx43 proteins and maintained cell-to-cell communication. Antiarrhythmic properties of VS and ACh were mediated via muscarinic receptors but were independent of heart rate deceleration. That cellular coupling can be improved through neural stimulation may lead to novel therapeutic strategies for preventing ventricular fibrillation during ischemia.
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Circulation. 2005;112:164-170; originally published online July 5, 2005; doi: 10.1161/CIRCULATIONAHA.104.525493
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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