Obligatory Role of Cardiac Nerves and $\alpha_1$-Adrenergic Receptors for the Second Window of Ischemic Preconditioning in Conscious Pigs


Abstract—We tested the hypothesis that cardiac nerves may mediate ischemic preconditioning. Pigs were chronically instrumented to measure aortic, left atrial and left ventricular pressures, and regional myocardial function (wall thickening). Hemodynamic variables, area at risk, and tissue blood flows (radioactive microspheres) were similar among groups. Myocardial infarct size following 60 minutes coronary artery occlusion and 4 days reperfusion, expressed as a fraction of the area at risk, was 42±4.0%, in innervated pigs and similar in pigs with regional cardiac denervation (CD, 41±2.5%). Infarct size in innervated pigs during the first window of preconditioning (first window) was markedly reduced (6±1.8%, $P<0.01$), as it was in the second window of preconditioning (second window) (16±3.3%, $P<0.01$). Although infarct size was still reduced in pigs with CD and first window preconditioning (49±1.8%, $P<0.01$), the protective effects of second window were abrogated in pigs with CD resulting in an infarct size of 38±5.6%. In another group of innervated pigs during pharmacological $\alpha_1$-adrenergic receptor (AR) blockade, infarct size was also not reduced during the second window (48±3.2%). Additionally, Western blot analysis of inducible nitric oxide synthase and cyclooxygenase-2 proteins demonstrated significant ($P<0.05$) upregulation following the second window in innervated pigs, but not in pigs with CD or $\alpha_1$-AR blockade. Thus, the mechanism of protection during the second window, but not the first window, appears to be dependent on cardiac nerves and $\alpha_1$-AR stimulation. (Circ Res. 2006;99:0-0.)

Key Words: myocardial infarction ■ sympathetic nervous system ■ nitric oxide ■ regional wall motion ■ coronary artery occlusion

Ischemic preconditioning is a mechanism by which a brief period of nonlethal ischemia protects the heart from subsequent lethal ischemia.1 Two distinct components of ischemic preconditioning have been described: classical, early preconditioning or the first window of preconditioning (first window),1 and late or second window of preconditioning (second window),2,3 each with distinct mechanisms.4–7 The goal of the current study was to determine the role of cardiac nerves in mediating ischemic preconditioning. Although studies have addressed this topic in the first window,8–13 very little attention has been paid to the role of cardiac nerves in mediating the second window. In part, this may be because of the requirement of the experimental design, which involves consecutive days of experiments, which are conducted best in vivo. Conversely, studies in isolated hearts, which are frequently used in preconditioning experiments, cannot be used because of both the nature of the protocol and the requirement for intact nerves in the control group.

A previous study, by our group, in conscious pigs indicated a protective role for innervation of the heart during acute myocardial ischemia and reperfusion.14 Our conclusion from the previous study was that denervation interfered negatively with the development and disposition of nitric oxide (NO) during ischemia and reperfusion. Since NO is an obligatory trigger mechanism for the development of late preconditioning,15 our interest focused on a potential role of cardiac nerves and NO in the second window. Accordingly, we examined the effects of regional cardiac denervation (CD) on the cardioprotection afforded by first and second window preconditioning during ischemia/reperfusion using a conscious, chronically instrumented pig model. Additionally, the effects of preconditioning were studied in the presence of pharmacological $\alpha_1$-adrenergic receptor (AR) or NO blockade.

Materials and Methods

Animals used in this study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (National
No Preconditioning

Group 1: Intact (n=5)  
Group 2: Cardiac denervation (n=5)

1st Window of Preconditioning

Group 3: Intact (n=5)  
Group 4: Cardiac denervation (n=4)

2nd Window of Preconditioning

Group 5: Intact (n=5)  
Group 6: Cardiac Denervation (n=5)  
Group 7: L-NNA (n=6)  
Group 8: α1-AR blockade (n=5)

Figure 1. The protocols used are depicted schematically.

Research Council, revised 1996) and the Institutional Animal Care  
and Use Committee, UMDNJ-New Jersey Medical School.

In Vivo Studies

Domestic swine, weighing 23.2±0.3 kg, were anesthetized and  
instrumented using aseptic technique as described previously.16 A  
miniature solid state pressure gauge was implanted in the left  
ventricle (LV) to measure LV pressure and dP/dt. Ultrasonic crystals  
were implanted to measure wall thickening in the potentially  
ischemic and nonischemic zones. A hydraulic occluder was im-  
planted on the left circumflex coronary artery (LCX) to induce  
coronary artery occlusion (CAO) and reperfusion (CAR). All pigs  
received preoperative intercostal nerve blocks (bupivicaine), mor-  
phine as a pre- and postoperative analgesic at 0.2 mg/kg IM, and  
fentanyl (75 g/hr) was then administered transdermally for 3 days.

All pigs were subjected to lethal ischemia, ie, 60 minutes of CAO  
followed by 4 days of CAR. In addition to control groups (no  
preconditioning), groups of pigs were subjected to either first  
window (2 episodes of 10 minutes CAO/10 minutes CAR), or second  
window (2 episodes of 10 minutes CAO/10 minutes CAR followed  
by 24 hour CAR). Groups of pigs were studied with intact innerva-  
tion or CD. The effects of preconditioning were also studied in the  
presence of 1) α1-AR blockade, either administered from the  
beginning of preconditioning through CAR the following day or just  
during the initial preconditioning stimuli, and 2) blockade of NO  
synthase. The number of pigs in each group, as well as all  
experimental protocols, is shown in the Figure and tables.

Regional CD of the posterior left ventricular free wall was accomplished using a  
combination of surgical and chemical techniques as described  
previously, and which resulted in a 99% reduction in tissue norepi-  
nephrine levels.14 Briefly, the LCX and the epicardial surface over  
the region of myocardium subtended by it were dissected carefully  
and phenol (88%) was then applied topically. For systemic  
1-AR blockade, prazosin (0.1 mg/kg, i.v.) was administered in pigs with  
intact cardiac innervation immediately before preconditioning ische-  
mia and either discontinued 1 hour after preconditioning (n=2) or  
continued during 24 hour reperfusion (n=3)(0.033 mg/kg/hr contin-  
uous i.v. infusion) before subsequent 60 minutes CAO and 4 days  
CAR. For systemic NO synthase blockade, N-nitro-L-arginine

| TABLE 1. Regional Myocardial Blood Flows During 55 minutes Coronary Occlusion (ml/min/g) |
|----------------------------------------------|---------------------------------|-----------------------------|--------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                                              | (n=3)                          | (n=3)                       | (n=4)                       | (n=4)                       | (n=6)                       | (n=4)                       | (n=6)                       | (n=4)                       |
| Ischemic Zone                               | Intact                         | Intact + Second Window      | CD                          | CD + Second Window          | L-NNA + Second Window       | α-blockade + Second Window  |
| Endo                                        | 0.1±0.0                        | 0.1±0.1                     | 0.1±0.04                    | 0.1±0.04                    | 0.1±0.1                     | 0.07±0.01                   |
| Epi                                         | 0.2±0.1                        | 0.1±0.02                    | 0.2±0.01                    | 0.1±0.04                    | 0.1±0.02                    | 0.10±0.02                   |
| Trans                                       | 0.1±0.04                       | 0.1±0.03                    | 0.1±0.02                    | 0.1±0.04                    | 0.1±0.04                    | 0.09±0.01                   |
| Non-Ischemic Zone                           | Intact                         |                              |                              |                             |                             |                             |
| Endo                                        | 1.4±0.1                        | 1.2±0.3                     | 1.3±0.4                     | 1.2±0.30                    | 1.2±0.2                     | 1.01±0.17                   |
| Epi                                         | 0.9±0.1                        | 0.9±0.2                     | 1.0±0.3                     | 0.9±0.20                    | 1.0±0.1                     | 0.95±0.19                   |
| Trans                                       | 1.1±0.1                        | 1.0±0.3                     | 1.1±0.4                     | 1.0±0.20                    | 1.1±0.1                     | 0.98±0.18                   |

CD = Cardiac Denervation; Endo = subendocardial; Epi = subepicardial; Trans = transmural.
(L-NNA)(35 mg/kg i.v.) was administered before both preconditioning and lethal ischemia.

The experiments were conducted 7 to 10 days after surgery. Diazepam was administered at 0.5 to 1.0 mg/kg for tranquilization before initiation of the experimental protocol and additionally as required, i.e., if the pig became agitated transiently. Hemodynamics were measured and recorded, and radioactive microspheres used to assess tissue blood flow at baseline and near the end of CAO, as described previously.14,17,18 In all pigs the CAO was induced by inflating the occluder to eliminate coronary blood flow during preconditioning or lethal ischemia followed by full reperfusion for either 10 minutes, 24 hours or 4 days, respectively.14,17,18 Hemodynamic data were recorded before and during preconditioning and lethal ischemia, throughout the first 3 hours and at 12 and 24 hours reperfusion (following both preconditioning or lethal ischemia), and 48, 72 and 96 hours after reperfusion following lethal ischemia (60 minutes CAO). Methods for defining the area at risk and evaluation of ischemic necrosis were similar to those described previously.14,17,18

Mortality
Mortality rates for all groups with second window preconditioning were not different ($\chi^2$). The mortality, 2 to 3 pigs in each group, occurred during the initial preconditioning stimulus and was because of ventricular fibrillation.

In Vitro Studies
Additional pigs were studied using similar protocols as those described above for in vitro analysis. Groups of pigs with and without CD, with and without second window preconditioning, or without CD with $\alpha_1$-AR blockade, were euthanized for tissue harvest 24 hours after the preconditioning stimulus (second window). The tissue samples were prepared for Western blot analysis for inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2).

Statistical Analysis
All data are expressed as mean ± SEM. Hemodynamic data at individual time points were analyzed using repeated measures ANOVA among the groups with a Student-Newman-Keuls test for posthoc comparison. $P<0.05$ was taken as the level for significance.

Results
Hemodynamic measurements in the groups of pigs for the second window studies are shown at baseline, at 60 minutes CAO, and at 4 days reperfusion in the online data supplement available at http://circres.ahajournals.org. CAO reduced myocardial blood flow transmurally. Near the end of occlusion (55 minutes CAO) the extent of reduction in blood flow in the ischemic region was confirmed using radioactive microspheres and was similar among groups (Table 1).

Second Window Preconditioning

Infarct Size
Infarct size (as a percent of the area at risk) is shown for all groups in Figure 2, and the size of the area at risk (as a percent of the LV and septum) in Table 2. Ischemic areas at risk were similar among groups (Table 2).

Infarct size in the area at risk at was significantly decreased, $P<0.05$, following second window preconditioning in pigs with intact innervation (16±3.3%) versus pigs with intact innervation without preconditioning (42±4.0%, Figure 2 and Table 2). However, infarct size was not reduced in pigs with CD and second window preconditioning (38±5.6%). After L-NNA and second window preconditioning, infarct size was also not reduced (40±6.1%) compared with that in innervated pigs with second window preconditioning without L-NNA. Infarct size in pigs with second window preconditioning and $\alpha_1$-AR blockade was also not reduced (48±3.2%) compared with innervated pigs with second window preconditioning without $\alpha_1$-AR blockade.

TABLE 2. Area at risk (AAR) and Infarct Size (IF)

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<td>21.5±1.1</td>
<td>9.1±1.8*</td>
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* Different from intact, $P<0.05$; CD = Cardiac Denervation; † AAR = Percent of LV and Septum at Risk; ‡ IF/AAR = Infarct Size as percent of Area at Risk.
Recovery of Regional Wall Thickening

The time course for recovery of regional function in the ischemic zone is shown in Figure 3. Ischemic zone wall thickening decreased similarly, i.e., became paradoxical during 60 minutes CAO in all groups studied. Functional recovery of ischemic zone wall thickening during reperfusion following 60 minutes CAO was significantly greater, *P* < 0.05, in pigs with intact innervation and second window preconditioning compared with pigs with intact innervation without second window preconditioning (Figure 3). Following 60 minutes CAO and 4 days reperfusion, ischemic zone wall thickening was less depressed, *P* < 0.05, in pigs with intact innervation and second window preconditioning (-46±6%) versus pigs with intact innervation without second window (-95±5%), (Figure 3). Regional function did not recover following second window preconditioning either in pigs with CD, systemic α-AR blockade, or systemic NO synthase blockade.

Western Blot Analysis: iNOS and COX-2

As expected, iNOS and COX-2 protein levels were significantly increased, *P* < 0.05, following second window preconditioning ischemia and 24 hours reperfusion in pigs with intact cardiac nerves and second window versus sham controls (Figure 4). This was not observed in pigs with CD or α-AR blockade administered during the initial preconditioning stimulus and second window preconditioning and 24 hour reperfusion (Figure 4).

First Window Preconditioning

Infarct Size

Infarct size following first window ischemic preconditioning was significantly reduced, *P* < 0.05, in both innervated pigs without CD (6±1.8%) and in pigs with CD (9±1.8%), compared with results in pigs either with CD (41±2.5%) or with innervation intact (42±4.0%) without first window protection (Figure 2 and Table 2).
Recovery of Regional Wall Thickening
Hemodynamics are shown in the online data supplement (available at http://circres.ahajournals.org), and the time course for recovery of regional ischemic zone function is shown in Figure 5. Functional recovery of ischemic zone wall thickening during reperfusion following first window preconditioning and 60 minutes CAO was similar in pigs without CD compared with pigs with CD (Figure 5) and also similar to innervated pigs with second window preconditioning (Figure 3). There were no significant differences in transmural blood flow reduction during CAO among the groups (data not shown).

Discussion
The current study demonstrated that the second window of preconditioning is abrogated following either regional CD or α1-AR blockade in conscious swine. The lack of delayed preconditioning in regionally denervated myocardium indicates that the presence of functional cardiac nerves and α1-AR activation are obligatory for the development of second window preconditioning. Interestingly, the cardiac nerves are not required for the first window. Additionally, the abrogation of second window preconditioning following denervation was associated with a lack of myocardial iNOS and COX-2 upregulation, both proteins being essential for the delayed preconditioning effect.4–6 This latter finding indicates that intact cardiac nerves are required for the preconditioning protection and for the protein synthesis of at least 2 obligatory mediators of second window cardioprotection, iNOS15,19–23 and COX-2.24

The role of cardiac nerves or α1-AR signaling has been studied extensively in the first window, with conflicting results. One study which utilized surgical sympathectomy failed to block first window preconditioning,8 consistent with the results in the current investigation. However, studies have found that norepinephrine depletion can either eliminate8 or not affect12,13 first window protection. Similarly, studies examining the role of α1-ARs have found either elimination of9,25 or no effect on10,11 first window preconditioning.

Importantly, no prior studies have examined the effects of CD on second window protection. The major finding of this current investigation demonstrated that intact cardiac nerves are essential for the development of second window protection, in spite of not being required to elicit first window protection with the same ischemic preconditioning stimuli. Furthermore, this delayed protection is conferred through α1-AR pathways. The latter finding, related to α1-AR mechanisms, is consistent with previous studies showing that exogenous α1-AR agonists can elicit delayed protection in numerous species, including mice,26 rats,27–29 rabbits,30–32 and humans.33 Of course, it must be appreciated that systemic α1-AR stimulation increases arterial pressure and activates a number of reflex pathways, which could be involved in mediating the preconditioning. These studies did not induce second window protection by a brief period of ischemia and did not block the effects of ischemia, since all the preconditioning interventions included pharmacological stimulation of α1-ARs.

One potential explanation for the divergent effects of denervation on the 2 phases of preconditioning, ie, first window and second window, in our study could be related to the distal molecular mechanisms involved in ischemic cardioprotection, for example, iNOS and COX-2 are critical mediators of second window protection, but not first window protection.3,6 CD prevented the upregulation of iNOS and COX-2 during second window in our model. Our finding that systemic NO synthase inhibition also abolished the beneficial effects of second window confirmed an obligatory role of NO for second window in our model. However, since these mechanisms are not involved in the first window,34 it is understandable that the early cardioprotection was not blocked. Accordingly, our study can be interpreted further to indicate that the cellular mechanisms mediating first window protection are not triggered by cardiac neural stimulation.

**Figure 5.** Effects of CAO and CAR on regional, ischemic zone wall thickening (IZWT) are shown for studies on the first window of protection in intact pigs (left panel), and CD pigs (right panel). In all groups CAO induced complete loss of regional wall motion. There was significant recovery of wall thickening over the 4 day period of study with first window protection in both innervated and CD pigs (closed squares). Baseline values and the number of animals are shown in parentheses. *P<0.05
Another experiment that demonstrates the differences between the role of cardiac nerves and $\alpha_1$-AR in mediating first and second window preconditioning, was the one in which $\alpha_1$-ARs were blocked only during the initial preconditioning stimuli. In these experiments, second window protection was also abrogated 24 hours later as was the upregulation of iNOS and COX2 at this time.

The finding that regional CD abrogates second window cardioprotection is consistent with results from our previous study which indicated a protective role for adrenergic innervation of the heart during acute myocardial ischemia and reperfusion, ie, the pigs with CD demonstrated more intense myocardial stunning and developed patchy subendocardial necrosis. It is conceivable that the delayed recovery of function following acute myocardial ischemia in that study is at least in part dependent on second window protection and was mediated by intact cardiac innervation.

The current investigation raises important questions for future studies, eg, the location of $\alpha_1$-AR activation and secondly the link between cardiac nerves and $\alpha_1$-AR activation and upregulation of iNOS and COX2. We believe it is unlikely that the protective effect involves an action on coronary vessels, since $\alpha_1$-AR regulation of coronary vessels is thought to be minimal in pigs, which is confirmed by our measurements of myocardial blood flow, ie, no differences in pigs with or without CD (data not shown). It is more likely that the protective effect occurs at the level of the myocyte. It is also unlikely that parasympathetic mechanisms mediate the effects observed in this study. First, the technique of CD used does not fully eliminate parasympathetic involvement, and secondly, selective $\alpha_1$-AR blockade with prazosin does not affect parasympathetic regulation, but did eliminate second window protection in this study. There are potentially additional mediators already described for second window preconditioning, which could also be involved. The contribution of the present study is to demonstrate the central role of cardiac nerves and $\alpha_1$-AR mediation of the upregulation of iNOS and COX2, which is required for second window protection. In conclusion, both the salutary effects on recovery of regional function and development of infarct size that are afforded by second window protection, are critically dependent on both intact cardiac nerves and $\alpha_1$-ARs. In contrast, the protection induced by the first window is not dependent on intact cardiac nerves.

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Disclosures
None.

References


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Hemodynamics and Non-Ischemic Regional Myocardial Function - Second Window

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**LV dP/dt (mmHg/sec)**

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* p<0.05 different from intact + p<0.05 different from baseline ‡ n=3 CD = Cardiac Denervation

Hemodynamics and Non-Ischemic Regional Myocardial Function - First Window

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<tr>
<th></th>
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<th>Baseline</th>
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<td>125 ±4</td>
<td>116 ±3</td>
</tr>
<tr>
<td>CD</td>
<td>5</td>
<td>97 ±3</td>
<td>86 ±3+</td>
<td>90 ±2</td>
<td></td>
<td>CD</td>
<td>5</td>
<td>123 ±5</td>
<td>127 ±11</td>
</tr>
<tr>
<td>CD+IPC</td>
<td>4</td>
<td>100 ±2</td>
<td>86 ±4+</td>
<td>100 ±6</td>
<td></td>
<td>CD+IPC</td>
<td>4</td>
<td>115 ±6</td>
<td>105 ±5</td>
</tr>
</tbody>
</table>

**LV dP/dt (mmHg/sec)**

<table>
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<th>n</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>5</td>
<td>3200 ±316</td>
<td>2530 ±255</td>
<td>2880 ±224</td>
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<td>Intact</td>
<td>5</td>
<td>3.75 ±0.45</td>
<td>4.18 ±0.47</td>
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<tr>
<td>Intact+IPC</td>
<td>5</td>
<td>3000 ±141</td>
<td>2520 ±361</td>
<td>3240 ±172</td>
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<td>Intact+IPC</td>
<td>5</td>
<td>4.55 ±0.43</td>
<td>4.62 ±0.31</td>
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<tr>
<td>CD</td>
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<td>2960 ±117</td>
<td>2560 ±194</td>
<td>2800 ±83</td>
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<td>CD</td>
<td>5</td>
<td>4.31 ±0.42</td>
<td>4.73 ±0.41</td>
</tr>
<tr>
<td>CD+IPC</td>
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<td>2800 ±141</td>
<td>2188 ±166+</td>
<td>2950 ±50</td>
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<td>CD+IPC</td>
<td>4</td>
<td>4.17 ±0.47</td>
<td>3.94 ±0.14</td>
</tr>
</tbody>
</table>

* p<0.05 different from intact + p<0.05 different from baseline IPC = Ischemic Preconditioning CD = Cardiac Denervation