Preservation of myofilament calcium responsiveness underlies protection against myocardial stunning by ischemic preconditioning

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Abstract

Objective: Whereas diminution of infarct size by ischemic preconditioning (IP) is well-accepted, protection against stunning is controversial. Since stunning is characterized by decreased myofilament Ca\(^{2+}\) responsiveness, we investigated whether IP would preserve myofilament responsiveness in a model of stunning.

Methods: Rat hearts were retrogradely perfused with Krebs–Henseleit (K–H) solution for 20 min and then subjected to 20 min of no-flow global ischemia, followed by 20 min of reperfusion in the absence (stunning) or in the presence (IP) of a previous 5-min period of ischemia followed by 15 min of reperfusion. A group of hearts perfused under non-ischemic conditions served as control. Thin ventricular trabeculae were dissected from each of the experimental groups and loaded with fura-2 to measure intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) and developed force.

Results: After 20 min of reperfusion, left ventricular developed pressure decreased in stunned hearts to 61±5% of control (\(P<0.01\)), whereas recovery was complete in the IP hearts (97±4%). Steady-state [Ca\(^{2+}\)]\(_i\)–force relationships revealed a decreased maximal Ca\(^{2+}\)-activated force in stunned hearts relative to control, but no change in the IP group. The Ca\(^{2+}\) required for 50% activation increased in stunning but not in IP.

Conclusions: These results show that the decrease in myofilament responsiveness that characterizes stunning is prevented by ischemic preconditioning.

Keywords: Ischemia; Reperfusion; Stunning; Calcium (cellular); Preconditioning

1. Introduction

Reperfusion after a brief episode of myocardial ischemia does not induce necrosis, but results in prolonged contractile dysfunction [1]. Such myocardial “stunning” is manifested clinically as sluggish recovery of pump function after coronary revascularization [2–4]. A decreased Ca\(^{2+}\) responsiveness of the myofilaments underlies stunning, perhaps as a consequence of Ca\(^{2+}\)-activated proteolysis during early reperfusion [5]. On the other hand, if the heart is pretreated with one or several brief episodes of ischemia, there can be paradoxical protection against subsequent lethal ischemic injury. This protective effect has been called ischemic preconditioning (IP) [6]. Preconditioning has been shown to be a powerful endogenous form of myocardial protection against infarction. Although infarction is not completely prevented, it is delayed, as the degree of protection is finite. The classical protection lasts for about 2–3 h [6–9] and has been called first window of protection. A delayed form of preconditioning or “second window of protection” was discovered in 1993 and exists between 24 and 72 h [10,11].

Myocardial infarction and stunning represent two very different types of injury and the effect of preconditioning cannot necessarily be extrapolated from one to the other.
Protection by preconditioning against myocardial infarction is widely accepted, but whether the protection extends to myocardial stunning is controversial [12-16]. Here we show, in a global ischemia/reperfusion rat heart model, that IP prevents stunning during the first window of protection. Since the cellular lesion in stunned myocardium resides at the level of the myofilaments and is characterized by a decreased Ca\(^{2+}\) responsiveness, we sought to determine whether these fundamental alterations of excitation-contraction coupling could be blunted by IP.

2. Methods

2.1. Whole rat hearts

In procedures that conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996), male rats (LBN-F1 strain, 200–250 g, Harlan, Indianapolis, IN, USA) were anesthetized by intraperitoneal injection of 0.2 to 0.3 ml sodium pentobarbital (6 grains/ml, ANPRO Pharmaceutical). According to previously described methodology [17], the heart was excised and retrogradely perfused at a constant rate of ~15 ml/min, with Krebs-Henseleit (K-H) solution equilibrated with a mixture of O\(_2\)-CO\(_2\) (95:5). The K-H solution was composed of (mmol/l) NaCl 120, NaHCO\(_3\) 20, KCl 5, MgSO\(_4\) 1.2, glucose 10, and CaCl\(_2\) 1.2, pH 7.35 to 7.40. The hearts were paced at 4.5 Hz, except for a period indicated below, via two wire electrodes placed on the right ventricle. Isovolumic left ventricular pressure was measured with an intracavitary balloon filled with water and connected to a pressure transducer. The volume of the balloon was adjusted to a diastolic pressure of ~10 mm Hg, which was kept constant for the whole experiment. The heart was placed in a water-jacketed container, to maintain a constant temperature of 37\(^\circ\)C. Temperature was monitored throughout the experiment by a probe inside the left ventricle. Fig. 1 shows a schematic representation of the perfusion protocols used in this work. All hearts were initially perfused for 10 min to allow stabilization of pressure development, and then were subjected to one of the following protocols: (1) Control group: the hearts were perfused continuously for 60 min (n=5). (2) Stunning group: the hearts were perfused for 20 min, and then subjected to 20 min of no-flow global ischemia, followed by 20 min of reperfusion (n=5). (3) Preconditioning group: the hearts were subjected to 5 min of no-flow global ischemia, followed by 15 min of reperfusion, and then another 20 min of no-flow global ischemia plus 20 min of reperfusion (n=5). For protocols 2 and 3, pacing was stopped after 3 min of ischemia, after which the hearts beat spontaneously, and resumed after 3 min of reperfusion.

2.2. Rat trabeculae

At the end of each protocol, the hearts were removed from the perfusion apparatus and subsequently perfused in procedures that conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996), male rats (LBN-F1 strain, 200–250 g, Harlan, Indianapolis, IN, USA) were anesthetized by intraperitoneal injection of 0.2 to 0.3 ml sodium pentobarbital (6 grains/ml, ANPRO Pharmaceutical). According to previously described methodology [17], the heart was excised and retrogradely perfused at a constant rate of ~15 ml/min, with Krebs-Henseleit (K-H) solution equilibrated with a mixture of O\(_2\)-CO\(_2\) (95:5). The K-H solution was composed of (mmol/l) NaCl 120, NaHCO\(_3\) 20, KCl 5, MgSO\(_4\) 1.2, glucose 10, and CaCl\(_2\) 1.2, pH 7.35 to 7.40. The hearts were paced at 4.5 Hz, except for a period indicated below, via two wire electrodes placed on the right ventricle. Isovolumic left ventricular pressure was measured with an intracavitary balloon filled with water and connected to a pressure transducer. The volume of the balloon was adjusted to a diastolic pressure of ~10 mm Hg, which was kept constant for the whole experiment. The heart was placed in a water-jacketed container, to maintain a constant temperature of 37\(^\circ\)C. Temperature was monitored throughout the experiment by a probe inside the left ventricle. Fig. 1 shows a schematic representation of the perfusion protocols used in this work. All hearts were initially perfused for 10 min to allow stabilization of pressure development, and then were subjected to one of the following protocols: (1) Control group: the hearts were perfused continuously for 60 min (n=5). (2) Stunning group: the hearts were perfused for 20 min, and then subjected to 20 min of no-flow global ischemia, followed by 20 min of reperfusion (n=5). (3) Preconditioning group: the hearts were subjected to 5 min of no-flow global ischemia, followed by 15 min of reperfusion, and then another 20 min of no-flow global ischemia plus 20 min of reperfusion (n=5). For protocols 2 and 3, pacing was stopped after 3 min of ischemia, after which the hearts beat spontaneously, and resumed after 3 min of reperfusion.

2.3. Measurement of intracellular \([Ca^{2+}]\) in trabeculae

[Ca\(^{2+}\)], was measured with fura-2, according to the method described by Backx and ter Keurs [20]. After 40–60 min of stabilization at 0.5 Hz stimulation frequency, pacing was stopped and fura-2 pentapotassium salt was microinjected iontophotically. After fura-2 loading, stimulation was resumed and [Ca\(^{2+}\)] was determined by epifluorescent illumination at 380 and 340 nm. The fluorescence was collected at 510 nm by a photomultiplier tube (R1527, Hamamatsu). The output of the photomultiplier was filtered at 100 Hz, collected by an analog-digital converter, and stored in a computer for later analysis. Intracellular Ca\(^{2+}\) was calculated by the following equation after subtraction of the corresponding background fluorescence of the trabeculae:
\[ [\text{Ca}^{2+}]_i = K'_d (R - R_{\text{max}})/(R_{\text{max}} - R) \]

where \( R \) is the observed ratio of fluorescence (340/380), \( K'_d \) is the apparent dissociation constant, \( R_{\text{max}} \) is the ratio 340/380 nm at saturating \([\text{Ca}^{2+}]_i\), and \( R_{\text{min}} \) is the ratio 340/380 nm at zero \([\text{Ca}^{2+}]_i\). The values of \( K'_d, R_{\text{max}} \) and \( R_{\text{min}} \) were 2.95, 9.55 and 0.50, respectively, as determined by in vivo calibration [17,18,20,21]. The apparent \( K'_d \) is the result of multiplying the true \( K'_d \) of fura-2 by a correction factor obtained from the ratio of fluorescence of the \( \text{Ca}^{2+} \)-free to \( \text{Ca}^{2+} \)-bound forms of fura-2 at 380 nm (Sf2/Sb2, see [22]).

2.4. Experimental protocols

In order to characterize excitation–contraction coupling, muscles were subjected to the following conventional experimental protocols [17,21]. We first studied the response to different extracellular calcium concentrations ([\( \text{Ca}^{2+} \)]_o) (0.5, 1.0, 1.5 and 2.0 mmol/l) during twitch contractions elicited by field stimulation (pulse duration 5 ms) at a rate of 0.5 Hz. Thereafter, the muscles were exposed to 5 \( \mu \)mol/l ryanodine for 30 min and stimulated periodically (once a min) at 10 Hz, to elicit tetani of 5–6 s duration. By varying \([\text{Ca}^{2+}]_i\), different levels of steady-state activation were achieved during tetani until maximal force was reached.

Steady-state \([\text{Ca}^{2+}]_i\)–force relationships were fitted with a function of the following form (Hill equation):

\[ F = F_{\text{max}} [\text{Ca}^{2+}]^n/(K_{50}^n + [\text{Ca}^{2+}]^n) \]

where \( F_{\text{max}} \) is the maximal \( \text{Ca}^{2+} \)-activated force, \( K_{50} \) is the [\( \text{Ca}^{2+} \)]_i required for 50% of maximal activation, and \( n \) is the Hill coefficient [17,21]. The Hill equation was fitted by an iterative method accordingly to the process of Levenberg–Marquardt with a tolerance of 0.05 (GRAPHPAD Prism™, version 1.0, GRAPHPAD Software).

2.5. Statistics

Student’s \( t \) test or one-way ANOVA (plus the post ANOVA Bonferroni \( t \) test for multiple comparisons) were used for statistical analysis of the data as appropriate [23]. A \( P \) value of less than 0.05 was considered to indicate significant differences between groups. Data are expressed as mean±S.E., unless otherwise indicated.

3. Results

3.1. Functional recovery of isovolumic left ventricular pressure in the stunning vs. preconditioning group

The starting point for this study was to determine to which extent the IP model used here might protect against stunning. The stunning protocol here was similar to that used in previous studies [17,24], and consisted of 20 min of total global ischemia followed by 20 min of reflow. The perfusion protocol for the IP model was essentially the same but with the addition of one brief cycle of ischemia-reflow (5 and 15 min respectively) before the long period of 20 min ischemia (see Fig. 1). We analyzed the functional recovery of these hearts. Fig. 2 shows the averaged left ventricular developed pressure, as percent of the corresponding pre-ischemic control, throughout the whole perfusion protocol in both the stunning (filled symbols) and the preconditioning (open symbols) groups. At the end of the reperfusion period, isovolumic left ventricular developed pressure (LVDP) had recovered completely in the preconditioning group (97±4% of control, NS), but in the stunning group it recovered only by 61±5% of control (\( P<0.05 \)). The asterisks in Fig. 2 indicate the significantly greater recovery of LVDP in the IP group vs. stunning, revealing full protection against stunning by IP.
slope in the stunning group (23 ± 7 mN/mm² [mmol/l]⁻¹, P < 0.05). Thus, these data show that, for a given change in [Ca²⁺], the change in peak systolic force is diminished in the stunned hearts, a finding consistent with previous reports and indicating a decrease in myofilament calcium responsiveness. This alteration, characteristic of the stunned myocardium, was blunted by IP. To distinguish whether the protection brought about by IP was due to preservation of the myofilament calcium sensitivity and/or to preservation of maximal force, we next determined the steady-state [Ca²⁺]–force relationships in the three experimental groups.

3.3. Steady-state [Ca²⁺]–force relationship in control, stunning and preconditioning group trabeculae

We performed steady-state activations of the muscles by tetanization [17,18,21]. Fig. 5 shows [Ca²⁺], and force during tetani in typical experiments from the three groups. The left panels of each row show two superimposed records of [Ca²⁺], (left) and force (right) obtained at two different [Ca²⁺], (one submaximal, 2.0 mmol/l, and the other maximal, 10.0 mmol/l). From such records we derived the [Ca²⁺]–force relationships that are presented in the right panel. The stunned muscle (middle row) generated less force than the control muscle (top row) at both submaximal and maximal activations, while the preconditioned muscle (bottom row) behaved like the control, with a remarkably preserved capacity to generate force.

The differences evident in Fig. 5 were observed consistently. Fig. 6 shows pooled data for steady-state activation from all muscles in the three groups (all groups n = 5). To facilitate the comparison between experimental groups, experimental curves in each group were normalized and then scaled by the averaged maximal force in that experimental group [17,18,21]. Thus, each curve plots the average absolute maximal force at saturating [Ca²⁺], in the corresponding experimental group; other data within each group are scaled relative to that group’s mean value. All points falling within 250 nmol/l wide bins of [Ca²⁺] were pooled to produce the ranges shown in each data point for [Ca²⁺], and force. Solid lines represent the best fit to a Hill equation of the mean force for each mean [Ca²⁺], value in each group. Once again, it is clear that IP protected against stunning, since the steady-state [Ca²⁺]–force relationship revealed a striking preservation of maximal force in this group (109 ± 6 control vs. 100 ± 10 mN/mm² preconditioning, P = NS), which was significantly higher than in the stunning group (100 ± 10 preconditioning vs. 56 ± 8 stunning, P < 0.05). On the other hand, no significant differences in the Hill coefficient n were observed among the three groups (5.73 ± 1.42, 5.89 ± 1.36 and 5.24 ± 0.55, control, stunning and preconditioning, respectively), but the whole curve in the preconditioning group was shifted to
Fig. 5. Steady-state contractile activation in typical experiments from the three groups. The left panel of each row shows two superimposed records of 
$[Ca^{2+}]_i$ (left) and force (right) during different tetanic activations at two $[Ca^{2+}]_i$ (2.0 mmol/l and 10.0 mmol/l). Points obtained at steady state from such records were used to construct the $[Ca^{2+}]_i$–force relationships shown in the right panel.

The left (i.e., to lower $[Ca^{2+}]_i$) compared to the stunning curve, revealing a full recovery of myofilament calcium sensitivity: the $[Ca^{2+}]_i$ required to activate 50% of the maximal force was not significantly different from that of the control group ($Ca_{50}$: 0.536±0.05 vs. 0.553±0.03 μmol/l, control vs. preconditioning, $P=NS$), and was
against stunning. This protection is attributable to both a
Our results highlight the preserved myofilament function
indicate a protective effect of ischemic preconditioning global ischemia in ferret [24] or rat hearts [34,36].
Pstunning,
the force of contraction at any given [Ca\(^+\)] relative to this interesting possibility. i
``preconditioned'' hearts. We found that stunning reduces ning; however, we have no experimental evidence to test
measure both [Ca\(^+\)] and force in muscles isolated from decreased calcium responsiveness produced by the stun-i
previous studies, and extend them by being the ®rst to of the contractile proteins [40], thus ``off-setting'' the
VARious studies have shown similar consequences of IP in transduction) remain to be elucidated. It is worth noting
developed pressure after 20 min of reperfusion (Fig. 2). tive roles (and those of other elements in IP signal
manifested by full recovery of the left ventricular de- observed mitigation of myofilament injury, but their rela-
Ca responsiveness. oxidant defense against reperfusion injury [39]. All of
this bene®cial effect is due to preservation of myofilament preservation of ATP stores, and an increase in the anti-
evaluate whether IP prevents stunning and, if so, whether the resulting effects are a decrease in calcium overload,
[17,24,26,27]. The goal of the present study was to dependent potassium channels in the mitochondria. Among
increased Ca responsiveness of the myofilaments seems to involve PKC activation and opening of ATP-
4. Discussion
Myocardial stunning has been characterized by a de-
controls during twitch contractions as well as during steady-state activation (con®rming Gao et al. [17]). De-
termination of the steady-state [Ca\(^+\)]–force relationships revealed a striking preservation of maximal force in the IP group, as well as full recovery of myofilament calcium sensitivity.
Gao et al. argued that partial proteolysis of the thin filament regulatory protein troponin I (TnI) underlies the contractile dysfunction of stunned myocardium [5]. The mitigation of stunning seen here as a consequence of IP would logically be predicted to involve a reduction of the amount of TnI proteolysis. We did not test this prediction in the present study.
There is no question that IP delays myocyte death and thereby limits infarct size. Since the original report by Murry et al. in 1986 [6], the protective effect of IP has been a constant ®nding among all laboratories and in every model and species in which the protection was evaluated. However, in contrast to the consensus among studies showing that IP limits infarct size, the protective effect against stunning is still controversial [12–16,31–34]. The important question to be answered is whether the improved contractile function after IP is secondary to a reduction in cellular necrosis or the result of protection of viable but stunned myocardium. The present model of stunning, with only 20 min of total global ischemia, shows no indication of tissue necrosis: cell–cell coupling remains robust (as gauged by the uniform spread of microinjected fura-2 salt throughout the stunned muscles), calcium cycling is virtually unaffected (Fig. 3 and [17]), and there is no contracture [17]. Consistent with these arguments, no significant degree of cell death was detected after 15–20 min of total global ischemia in ferret [24] or rat hearts [34,36].
Our results highlight the preserved myofilament function as evidence of preconditioning against stunning, but they do not pinpoint the mechanism of the protection. Calcium overload and free radical generation are the two main mechanisms by which the stunned myocardium develops (see [35] for review). These two mechanisms, which are undoubtedly connected [37,38], induce a series of events leading to the decrease in myofilament responsiveness [17,24,26,27]. The mechanism of protection induced by IP seems to involve PKC activation and opening of ATP-dependent potassium channels in the mitochondria. Among the resulting effects are a decrease in calcium overload, preservation of ATP stores, and an increase in the anti-

![Graph](image)

Fig. 6. Pooled data for steady-state contractile activation in control, stunning and preconditioning groups. Tetani were stimulated in individual muscles from each group at various extracellular calcium concentrations ([Ca\(^+\)]), and the steady values of [Ca\(^+\)], and force were quantified. Pooled data represent means±S.E. of all data points in each group which fell within bins of 250 nmol/l width (0–249 nmol/l, 250–499 nmol/l, etc.). Mean maximal force was calculated in each group, and the pooled data in each group were scaled appropriately. For a better comparison among groups we superimposed (solid lines) the best fit to a Hill equation of the mean force for each mean [Ca\(^+\)], value in each group.

significantly lower than in the stunning group (Ca\(_{501}\): 0.553±0.03 vs. 0.791±0.09 μmol/l, preconditioning vs. stunning. P<0.05). The results presented here clearly indicate a protective effect of ischemic preconditioning against stunning. This protection is attributable to both a preserved maximal Ca\(^+\)-activated force and a preserved myofilament Ca\(^+\) sensitivity.

4. Discussion
Myocardial stunning has been characterized by a de-
cribed Ca\(^+\) responsiveness of the myofilaments [17,24,26,27]. The goal of the present study was to evaluate whether IP prevents stunning and, if so, whether this beneficial effect is due to preservation of myofilament Ca\(^+\) responsiveness.
We found a strong protection against stunning by IP, as manifested by full recovery of the left ventricular de-
veloped pressure after 20 min of reperfusion (Fig. 2). Various studies have shown similar consequences of IP in a variety of species [28–30]; our results con®rm those previous studies, and extend them by being the ®rst to measure both [Ca\(^+\)], and force in muscles isolated from “preconditioned” hearts. We found that stunning reduces the force of contraction at any given [Ca\(^+\)], relative to controls during twitch contractions as well as during steady-state activation (con®rming Gao et al. [17]). Determination of the steady-state [Ca\(^+\)]–force relationships revealed a striking preservation of maximal force in the IP group, as well as full recovery of myofilament calcium sensitivity.
References


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