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Demonstration of an early and a late phase of ischemic preconditioning in mice

YIRU GUO, WEN-JIAN WU, YUMIN QIU, XIAN-LIANG TANG, ZEQUAN YANG, and ROBERTO BOLLI

Experimental Research Laboratory, Division of Cardiology, University of Louisville, Louisville, Kentucky 40292

Abstract

It is unknown whether ischemic preconditioning (PC; either early or late) occurs in the mouse. The goal of this study was to answer this question and to develop a reliable and physiologically relevant murine model of both early and late ischemic PC. A total of 201 mice were used. In nonpreconditioned open-chest animals subjected to 30 min of coronary occlusion followed by 24 h of reperfusion, infarct size (tetrazolium staining) averaged 52% of the region at risk. When the 30-min occlusion was performed 10 min after a PC protocol consisting of six cycles of 4-min occlusion and 4-min reperfusion, infarct size was reduced by 75%, indicating an early PC effect. When the 30-min occlusion was performed 24 h after the same PC protocol, infarct size was reduced by 48%, indicating a late PC effect. In mice in which the 30-min occlusion was followed by 4 h of reperfusion, infarct size was similar to that observed after 24 h of reperfusion, indicating that a 4-h reperfusion interval is sufficient to detect the final extent of cell death in this model. Fundamental physiological variables (body temperature, arterial oxygenation, acid-base balance, heart rate, and arterial pressure) were measured and found to be within normal limits. Taken together, these results demonstrate that, in the mouse, a robust infarct-sparing effect occurs during both the early and the late phases of ischemic PC, although the early phase is more powerful. This murine model is physiologically relevant, provides reliable measurements, and should be useful for elucidating the cellular mechanisms of ischemic PC in genetically engineered animals.

Keywords

coronary occlusion; coronary reperfusion; transgenic animals; knockout animals; genetic manipulations

Ischemic preconditioning (PC) is a powerful cardioprotective mechanism that confers relative resistance against myocellular death resulting from ischemia-reperfusion injury (1, 13, 16, 31, 33). The time course of ischemic PC is characterized by an immediate but short-lived wave of protection (early phase of PC) (1, 11, 13, 14, 16, 33, 38) followed, 12–24 h later, by a second, sustained window of protection that lasts at least 72 h (late phase of PC) (3–6, 9, 10, 27, 29–31, 36, 38, 45, 46, 48, 49, 55). Because of its remarkable efficacy, there is considerable interest in exploiting ischemic PC to develop therapeutic strategies that can enhance the tolerance of the heart to ischemic injury in patients with coronary artery disease (8, 13, 16, 31). Clinical application of ischemic PC, however, will require a detailed understanding of the molecular and cellular mechanisms underlying this endogenous adaptive phenomenon.

It is now apparent that activation of cellular kinases (e.g., protein kinase C and mitogen-activated protein kinases) plays an important role in both the early and the late phases of ischemic PC (3, 6, 13, 15, 16, 23, 31, 36, 37, 53, 56) and that upregulation of cardioprotective genes underlies the development of late PC (10, 30, 31, 40, 48). The specific isoforms of kinases involved, however, remain to be identified. Similarly, definitive evidence for a cause-and-effect relationship between the activity of a specific gene and the development of late PC is still lacking. Solving these issues with pharmacological approaches would be difficult because most inhibitors are not entirely specific and do not completely inhibit the target kinase or transcription factor. In contrast, genetic manipulations that either overexpress or disrupt a gene (transgenesis and gene targeting) can provide conclusive demonstration of the causative role of a gene product in ischemic PC. Thus the use of transgenesis and gene targeting for interrogating the function of individual proteins would be a powerful approach to investigating the mechanism of ischemic PC.

The mouse is the species commonly used for transgenesis and gene targeting. Although transgenesis is theoretically possible in larger mammals (e.g., rabbits or pigs), developing such models would be extremely costly and would require considerable time, making these models impractical. Furthermore, gene targeting has not been reported thus far in species other than the mouse. Thus, to utilize both transgenesis and gene targeting to study ischemic PC, it is necessary to use mice. Murine models of myocardial infarction have been developed in previous studies (22, 32). However, to our knowledge, it is unknown whether ischemic PC (either early or late) occurs in the mouse. This issue is particularly important with respect to the late phase of ischemic PC, which has been suggested to be species dependent because it has been observed in dogs (27) and rabbits (3–5, 30, 38, 48, 55) but not in pigs (39) or rats (24). Furthermore, the unique technical challenges associated with inducing infarctions in mice raise the concern that the results obtained in this model may not be as reliable and physiological as those obtained in larger species, in which the margin for error is wider and physiological parameters can be measured more easily.

Accordingly, the goals of the present study were 1) to determine whether the early and the late phases of ischemic PC exist in the mouse; 2) if so, to compare their relative potencies; 3) to establish whether the magnitude of the PC protection can be accurately assessed with reperfusion intervals as short as 4 h or whether survival surgery (24 h of reperfusion) is required; and 4) to develop reliable murine models of early and late PC in which fundamental physiological variables (body temperature, oxygenation, acid-base balance, heart rate, and arterial blood pressure) are carefully controlled and kept within normal values.

METHODS

This study was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Louisville School of Medicine and with the *Guide for the Care and Use of Laboratory Animals* [DHHS Publication No. (NIH) 86–23].

Experimental preparation

Male ICR (Institute of Cancer Research) mice (weight 33.3 ± 0.5 g; age 8–12 wk) were obtained from Harlan Sprague Dawley (Houston, TX) and housed under specific pathogen-free conditions in a room with a 24°C temperature, 55–65% relative humidity, and a 12:12-h light-dark cycle. Mice were premedicated with atropine sulfate (0.04 mg/kg im) and anesthetized 5 min later with pentobarbital sodium (50 mg/kg ip). Additional doses of pentobarbital were given during the protocol as needed to maintain anesthesia. The animals were placed in a supine position with the paws taped to the operating table. Surface leads were placed subcutaneously to obtain the electrocardiogram (ECG), which was recorded

throughout the experiments on a thermal array chart recorder (Gould TA6000). Before surgery started, mice were given gentamicin (0.7 mg/kg im).

A midline cervical skin incision was performed, and the muscles overlying the trachea were reflected to allow visualization of the endotracheal tube (PE-60 tubing) as it was placed in the trachea. To facilitate intubation, a rubber band was placed behind the upper incisors and fastened to the operating table so that the neck was slightly extended. To place the endotracheal tube, the tongue was slightly retracted, and the beveled end of the tube (which was marked with a black marker) was inserted through the larynx and into the trachea with care taken not to puncture the trachea or other structures in the pharyngeal region. The tube was advanced 8–10 mm from the larynx and taped in place to prevent dislodgment. The animals were ventilated with room air supplemented with oxygen (2 l/min) at a rate of 105 breaths/min and with a tidal volume of 2.1–2.5 ml using a rodent ventilator (Harvard Apparatus, South Natick, MA). The endotracheal tube was inserted loosely into the tube connected to the ventilator so as to avoid lung overexpansion. A catheter was inserted into the external jugular vein for fluid infusion. In selected studies, a catheter was inserted into the carotid artery for measurement of blood pressure (DTX pressure transducer, Viggo-Spectramed, Oxnard, CA) and analysis of blood gases. To replace blood losses, blood from a donor mouse was given intravenously at a dose of 40 ml/kg (~1 ml) divided into three equal boluses (first bolus, after the endotracheal tube was connected to the ventilator; second bolus, after the chest was opened; third bolus, after the chest was closed). Body temperature was carefully monitored with a rectal probe connected to a digital thermometer (Cole-Parmer Instrument, Vernon Hill, IL) and was maintained as close as possible to 37.0°C throughout the experiment by using a heating pad and heat lamps.

With the aid of a dissecting microscope (Fisher Scientific, Pittsburgh, PA) and a microcoagulator (ASSI Polar-Mate Isolator, San Diego, CA), the chest was opened through a midline sternotomy. An 8-0 nylon suture was passed with a tapered needle under the left anterior descending coronary artery 2–3 mm from the tip of the left auricle, and a nontraumatic balloon occluder was applied on the artery. Coronary occlusion was induced by inflating the balloon occluder. Successful performance of coronary occlusion and reperfusion was verified by visual inspection (i.e., by noting the development of a pale color in the distal myocardium on inflation of the balloon and the return of a bright red color due to hyperemia after deflation) and by observing S-T segment elevation and widening of the QRS on the ECG during ischemia and their resolution after reperfusion. After the coronary occlusion-reperfusion protocol was completed, the chest was closed in layers, and a small catheter was left in the thorax for 10–20 min to evacuate air and fluids. The mice were removed from the ventilator, kept warm with heat lamps, given fluids (1.0–1.5 ml of 5% dextrose in water intraperitoneally), and allowed 100% oxygen via nasal cone.

Experimental protocol

Ischemic PC was produced with a sequence of six cycles of 4-min coronary occlusion and 4-min reperfusion (Fig. 1). This protocol was selected because it has proved highly effective in inducing late PC in rabbits (38, 48). Myocardial infarction was produced by a 30-min coronary occlusion followed by either 4 or 24 h of reperfusion. A 30-min occlusion was selected because in pilot studies it produced infarcts averaging ~50% of the risk region in control animals, which enabled us to detect either a detrimental or a beneficial effect of the intervention examined.

Eight groups of mice were studied (Fig. 1). Mice in *group I* (control group, 4-h reperfusion) were subjected to 30 min of coronary occlusion followed by 4 h of reperfusion. To assess whether the duration of reperfusion affects infarct size, *group II* (control group, 24-h reperfusion) was subjected to 30 min of occlusion followed by 24 h (instead of 4 h) of

reperfusion. To assess the protective effects of the early phase of PC, mice in *group IV* (early PC group) underwent six cycles of 4-min occlusion and 4-min reperfusion followed, 10 min later, by 30 min of coronary occlusion and 24 h of reperfusion. *Group III* (early PC sham group) served as the control for *group IV*; in these mice, the chest was opened for 1 h (interval corresponding to the duration of the sequence of six cycles of 4-min occlusion and 4-min reperfusion in *group IV*) before 30 min of occlusion followed by 24 h of reperfusion. To assess the protective effects of the late phase of PC, mice in *groups VI* and *VIII* underwent a sequence of six cycles of 4-min occlusion and 4-min reperfusion on *day 1*. The chest was then closed, and the animals were allowed to recover. Twenty-four hours later, the mice were reanesthetized, the chest was reopened, and the 8-0 nylon suture (which had been left in place after the first surgery) was used to apply a balloon occluder and to produce 30 min of coronary occlusion followed by reperfusion for either 4 [*group VI* (late PC group, 4-h reperfusion)] or 24 h [*group VIII* (late PC group, 24-h reperfusion)]. To determine whether the surgical trauma has any impact on infarct size 24 h later, on *day 1* mice in *group V* (late PC sham group, 4-h reperfusion) and *group VII* (late PC sham group, 24-h reperfusion) were subjected to the same surgical protocol as *groups VI* and *VIII* (open-chest state for 60 min with placement of the 8-0 nylon suture) but did not undergo coronary occlusion; 24 h later, the mice underwent 30 min of occlusion followed by 4 (*group V*) or 24 h of reperfusion (*group VII*).

Postmortem tissue analysis

At the conclusion of the study, the mice were given heparin (1 U/g ip), after which they were anesthetized with pentobarbital sodium (35 mg/kg iv) and euthanized with an intravenous bolus of KCl. The heart was excised and perfused with Krebs-Henseleit solution through an aortic cannula (22- or 23-gauge needle) using a Langendorff apparatus. To delineate infarcted from viable myocardium, the heart was then perfused with a 1% solution of 2,3,5-triphenyltetrazolium chloride in phosphate buffer (pH 7.4, 37°C) at a pressure of 60 mmHg (~3 ml over 3 min). To delineate the occluded-reperfused coronary vascular bed, the coronary artery was then tied at the site of the previous occlusion and the aortic root was perfused with a 5% solution of phthalo blue dye (Heucotech, Fairless Hill, PA) in normal saline (2 ml over 3 min). As a result of this procedure, the portion of the left ventricle (LV) supplied by the previously occluded coronary artery (region at risk) was identified by the absence of blue dye, whereas the rest of the LV was stained dark blue (Figs. 2–4). The heart was frozen, after which all atrial and right ventricular tissues were excised. The LV was cut into 5–7 transverse slices, which were fixed in 10% neutral buffered formaldehyde and, 24 h later, weighed and photographed (Nikon AF N6006). The transparencies were projected onto a paper screen at $\times 30$ magnification, and the borders of the infarcted, ischemic reperfusion, and nonischemic regions were traced. The corresponding areas were measured by computerized videoplanimetry (Adobe Photo-shop, version 4.0), and from these measurements infarct size was calculated as a percentage of the region at risk using methods analogous to those employed in previous studies (2, 35, 38, 39, 48).

Statistical analysis

Data are reported as means $\pm$ SE. Heart rate and body temperature were analyzed with a two-way repeated-measures ANOVA (time and group). Infarct size and risk region size were analyzed with a one-way ANOVA followed by unpaired Student's *t*-tests with the Bonferroni correction (51). The relationship between infarct size and risk region size was compared among groups with an analysis of covariance (ANCOVA), with the size of the risk region as the covariate (38). The correlation between infarct size and risk region size was assessed by linear regression analysis using the least-squares method. All statistical analyses were performed using the SAS software system (41). Two-way ANOVA was performed using the general linear models procedure (41).

RESULTS

A total of 201 mice were used in this investigation (47 for the pilot studies and 154 for the studies of ischemic PC).

Pilot studies

Initially, we induced anesthesia with xylazine (7.5 mg/kg im) and ketamine (55 mg/kg im); however, we found that the heart rate was quite low (280–330 beats/min), which was clearly nonphysiological. We therefore chose pentobarbital anesthesia, as used by Michael et al. (32). After the anesthetic was selected, a series of pilot studies was performed in 47 mice. First, we sought to establish physiological parameters to be used as a reference for subsequent experiments. In eight mice, ECG leads were placed subcutaneously and the animals allowed to recover. Heart rate was monitored in the conscious state on the following days and was found to average 668 ± 31 beats/min (range 490–760 beats/min). In 16 pentobarbital-anesthetized mice, mean arterial blood pressure before thoracotomy was found to average 97.2 ± 4.4 mmHg. In 39 pentobarbital-anesthetized mice, rectal temperature before thoracotomy was found to average $37.0 \pm 0.4^\circ\text{C}$. In subsequent studies, the experimental conditions were adjusted to maintain heart rate, arterial blood pressure, and body temperature as close as possible to these values.

Another series of pilot studies was performed to identify the optimal ventilatory parameters. Because the endotracheal tube used was without a cuff, the tidal volume was adjusted by observing the inflation of the lungs after the chest was opened. We found that an average tidal volume of 2.2 ± 0.1 ml resulted in adequate inflation of the lungs without overexpansion. With the use of this tidal volume, different ventilatory rates were tested in 23 open-chest mice and arterial blood gases were analyzed in each animal (Table 1). The results showed that even small changes in ventilatory rate resulted in significant changes in arterial blood gases (Table 1), emphasizing the importance of these measurements. A ventilatory rate of 105 breaths/min was found to produce optimal values of arterial PO_2 , PCO_2 , and pH, as detailed in Table 1. This rate is within the range observed in spontaneously breathing mice (25, 54). Accordingly, this rate was used in the present study.

Additional pilot studies were performed to measure arterial blood pressure in mice subjected to open-chest surgery. The results are summarized in Fig. 5. In five mice, one dose of blood (13.3 ml/kg; ~0.4 ml) was given immediately after the thoracotomy in an effort to prevent hypotension. Despite this, mean arterial blood pressure fell to 63.4 ± 3.7 mmHg after the chest was opened (probably due to the loss of negative intrathoracic pressure and to the positive end-expiratory pressure) (Fig. 5). Furthermore, after the chest was closed, another drop in blood pressure was noted, to a nadir of 63.0 ± 9.7 mmHg (Fig. 5). Because these hypotensive episodes could induce ischemic PC, we decided to administer three doses of blood (instead of one). The first dose was given before the chest was opened, the second immediately after the chest was opened, and the third after the chest was closed. Each dose consisted of 13.3 ml/kg (~0.4 ml). With this protocol, mean arterial pressure remained ~80 mmHg throughout a 1-h period of open-chest state (Fig. 5). Next, we tested whether this protocol of fluid replacement was sufficient to prevent severe hypotension in mice undergoing the sequence of six coronary occlusion-reperfusion cycles in which myocardial ischemia would be expected to cause a further drop in arterial pressure. Although each coronary occlusion caused a drop in arterial pressure, the three doses of blood resulted in mean arterial pressure being maintained ~80 mmHg throughout the six occlusion-reperfusion cycles (Fig. 5). Thus, with the fluid supplementation protocol detailed above and with careful precautions taken to minimize blood losses, arterial blood pressure could be kept at adequate levels throughout the six occlusion-reperfusion cycles.

Exclusions

A total of 154 mice were used for the studies of ischemic PC. Twenty-one mice died because of hemorrhage ($n = 5$), pneumothorax ($n = 6$), pentobarbital overdose ($n = 1$), or other reasons ($n = 9$). Five mice died of ventricular fibrillation or presumed arrhythmias or heart failure during coronary occlusion or during the first 2 h of reperfusion: one in *group I* (control, 4-h reperfusion), one in *group II* (control, 24-h reperfusion), one in *group III* (early PC sham), and two in *group VI* (late PC, 4-h reperfusion). Thirty-six mice (23%) were excluded because of technical problems, including body temperature out of normal range ($n = 2$), malfunction of the ventilation system ($n = 5$), damage to the coronary vessels ($n = 6$), inadequate postmortem staining ($n = 5$), balloon malfunction ($n = 13$), and postoperative complications or other technical problems ($n = 5$). Ninety-two mice successfully completed the entire protocol and were included in the analysis of region at risk and infarct size. Thus total mortality (surgical mortality plus mortality due to coronary occlusion-reperfusion) was 17% (26 of 154).

Body temperature and heart rate

Rectal temperature was controlled strictly throughout the experiment with the use of heat lamps and a heating pad. As a result, temperature remained within a narrow, physiological range ($36.4\text{--}37.6^{\circ}\text{C}$) in all groups (Table 2). Heart rate remained stable throughout the protocol in each group (Table 2). Although in some groups the heart rate was 10–20% lower than the average heart rate measured in the pilot studies in conscious mice (668 ± 31 beats/min), it was still within the range of measurements obtained in these pilot studies ($490\text{--}760$ beats/min). Heart rate did not differ significantly among the four groups in which the 30-min coronary occlusion was performed on *day 1* (*groups I–IV*). In the four groups in which the 30-min coronary occlusion was performed on *day 2* (*groups V–VIII*), the heart rate was 10–20% higher than the corresponding values measured on *day 1* in *groups I–IV* (Table 2), possibly reflecting the effect of the surgical trauma 24 h earlier. However, there was no statistically significant difference among *groups V–VIII*.

Region at risk and infarct size

There were no significant differences among the eight groups with respect to LV weight or weight of the region at risk (Table 3). In *group I*, the 30 min of coronary occlusion followed by 4 h of reperfusion resulted in an infarct size of $50.9 \pm 2.6\%$ of the region at risk (Fig. 6). Similar results were obtained in *group II*, which underwent 30 min of coronary occlusion and 24 h of reperfusion ($53.2 \pm 3.6\%$ of the region at risk) (Fig. 6), indicating that the assessment of cell death at 4 h represents the final extent of myocardial infarction in this model. A representative example of the infarctions observed in *group II* is shown in Fig. 2. The large, confluent areas of infarction spanning most of the thickness of the LV wall, with thin rims of viable subendocardial tissue, were characteristic of all five nonpreconditioned groups (*groups I–III*, *V*, and *VII*).

In *group III* (early PC sham group), keeping the chest open for 60 min before the 30-min coronary occlusion had no effect on infarct size ($56.7 \pm 2.4\%$ of the region at risk vs. $53.2 \pm 3.6\%$ in *group II*) (Fig. 6). However, a sequence of six cycles of 4-min occlusion and 4-min reperfusion ending 10 min before the 30-min occlusion (*group IV*, early PC group) dramatically reduced infarct size to $14.2 \pm 1.9\%$ of the region at risk, indicating a powerful early PC effect against infarction (Fig. 6). Early PC in *group IV* decreased infarct size by an average of 75% compared with that in *group III*. A representative example of the infarctions noted in *group IV* is shown in Fig. 3. In contrast to the confluent, homogeneous areas of infarction noted in nonpreconditioned hearts (Fig. 2), in *group IV* only small, sporadic areas of cell death were noted.

In *groups V* and *VII* (late PC sham groups, 4-h and 24-h reperfusion), infarct size (49.4 ± 3.4 and $49.9 \pm 4.0\%$ of the region at risk, respectively) was indistinguishable from that in *groups I* and *II* (control groups, 4-h and 24-h reperfusion) (Fig. 6), indicating that a thoracotomy with a 60-min period of open-chest state without coronary occlusion did not affect the extent of cell death induced by a 30-min coronary occlusion 24 h later. However, when mice were preconditioned with six cycles of 4-min coronary occlusion and 4-min reperfusion on *day 1* [*groups VI* (late PC group, 4-h reperfusion) and *VIII* (late PC group, 24-h reperfusion)], the size of the infarct produced by a 30-min coronary occlusion 24 h later (*day 2*) was reduced to 22.2 ± 2.6 and $25.9 \pm 3.3\%$ of the region at risk, respectively; these values were significantly smaller ($P < 0.05$) than the corresponding values in sham-preconditioned mice (49.4 ± 3.4 and $49.9 \pm 4.0\%$ of the risk region in *groups V* and *VII*, respectively) (Fig. 6), indicating the development of a late PC effect. Late PC in *groups VI* and *VIII* decreased infarct size by an average of 55 and 48%, respectively, compared with *groups V* and *VII*. Thus the magnitude of the late PC effect against infarction was similar after 4 h of reperfusion (*groups VI* and *V*) and after 24 h of reperfusion (*groups VIII* and *VII*), indicating that a 4-h reperfusion interval was sufficient to detect the full extent of myocardial salvage afforded by late PC. A representative example of the infarctions observed in *group VIII* is shown in Fig. 4. Patchy areas of infarction were noted instead of the confluent infarctions seen in nonpreconditioned hearts (Fig. 2). This patchy pattern of cell death was characteristic of both of the two late PC groups (*groups VI* and *VIII*). In *group VIII* (late PC, 24-h reperfusion), infarct size was significantly ($P < 0.05$) greater than in *group IV* (early PC, 24-h reperfusion) (Table 3 and Fig. 6), indicating that, in the mouse, the early phase of ischemic PC affords greater protection than the late phase.

In all eight groups, the size of the infarction was positively and linearly related to the size of the region at risk ($r = 0.87, 0.77, 0.85, 0.77, 0.87, 0.64, 0.91$, and 0.16 in *groups I–VIII*, respectively) (Fig. 7). The regression line, however, was shifted to the right in *group IV* compared with *group III* ($P < 0.05$ by ANCOVA) (Fig. 7, *middle*) and in *groups VI* and *VIII* compared with *groups V* and *VII*, respectively ($P < 0.05$ by ANCOVA for both) (Fig. 7, *right*), indicating that, for any given size of the region at risk, the resulting infarction was smaller in preconditioned than in control mice.

The intraobserver and interobserver variabilities in the measurements of infarct size were carefully determined. When the same observer measured infarct size twice, there was $<3\%$ variability. When two different observers (Y. Guo and R. Bolli) calculated infarct size without knowledge of each other's assessment, the correlation coefficient was found to be 0.90 and the differences $<5\%$ ($n = 30$ mice). These data demonstrate that the measurements of infarct size in our mouse model are highly reproducible.

DISCUSSION

The purpose of this study was to develop a reliable and physiologically relevant model of ischemic PC that can be used in genetically engineered animals. Our results can be summarized as follows: 1) in the mouse, a sequence of six cycles of 4-min coronary occlusion and 4-min reperfusion induces a powerful infarct-sparing effect during both the early and the late phases of ischemic PC; 2) the magnitude of the protection afforded by the early phase of PC ($\sim 75\%$ reduction in infarct size) is greater than that afforded by the late phase of PC ($\sim 48\text{--}55\%$ reduction in infarct size); 3) in both nonpreconditioned and preconditioned mice, the size of the infarct is similar after 4 and 24 h of reperfusion following the 30-min occlusion, indicating that a 4-h reperfusion interval is sufficient to assess ischemic PC in this model; 4) despite the small size of the mouse, it is possible to study ischemic PC in this model under conditions in which basic physiological variables (body temperature, arterial oxygenation, acid-base balance, heart rate, and arterial blood

pressure) are kept within normal limits; and 5) both the quality of the postmortem staining for region at risk and infarction and the reproducibility of the measurements of infarct size are excellent and compare favorably with those in larger species.

Previous studies have demonstrated that myocardial infarction can be produced and quantitated in mice (22, 32). To our knowledge, this is the first study to demonstrate that ischemic PC (either early or late) exists in the mouse. This murine model of early and late PC should be useful for investigating the impact of genetic manipulations on physiological end points in vivo. By applying this model to mice with overexpression or targeted disruption of individual genes implicated in the cellular pathways underlying ischemic PC, it should be possible to conclusively establish the role of a specific gene product in the genesis of PC in the intact animal.

Physiological relevance of model

A major concern in the design of these experiments was to ensure that the results would be physiologically relevant. The minuscule size of the murine heart necessitates miniaturization of the procedures used in larger species and therefore poses a unique challenge in terms of maintaining general experimental conditions within normal values and avoiding artifacts. In the present study, a considerable amount of preliminary work (summarized in *Pilot studies*) was performed before ischemic PC was investigated. Because temperature is a major determinant of infarct size (12, 17, 20, 43), this variable was tightly controlled throughout the experiment by using heating pads and heat lamps while continuously monitoring rectal temperature. Our results demonstrate that, with the use of these procedures, temperature was kept within a narrow range (36.4–37.6°C; Table 2) that represents the normal range for the mouse (25, 44), as confirmed by our pilot studies, in which rectal temperature averaged $37.0 \pm 0.4^\circ\text{C}$. Hypoxemia, acidosis, and alkalosis may also have a major influence on animal survival, infarct size, and/or ischemic PC. Accordingly, we measured arterial pH, P_{O_2} , P_{CO_2} , and bicarbonate levels in mice subjected to open-chest surgery (Table 1). These measurements demonstrated that, with a ventilatory rate of 105 breaths/min and an average tidal volume of 2.2 ml, all parameters were within the physiological range for the mouse (18); in particular, arterial pH was kept at ~ 7.40 and adequate oxygenation was maintained throughout the open-chest state (Table 1). Careful control of blood gases is important in the mouse, because small variations in ventilatory rate result in marked variations in arterial blood gases (Table 1).

Heart rate and arterial pressure are important indexes of normal cardiovascular homeostasis and are also important determinants of the severity of myocardial ischemia. As elaborated in RESULTS, we avoided anesthesia with ketamine-xylazine because these agents resulted in unacceptably low heart rates (280–330 beats/min), clearly outside of the physiological range, which in our pilot studies in conscious mice was found to be 490–760 beats/min (average 688 ± 31 beats/min). With the use of pentobarbital anesthesia, the heart rates recorded in the present experiments (Table 2) were reasonably close to those measured in conscious mice in our pilot studies and in prior studies (18, 25, 28, 42, 44, 47, 54). The blood volume of a 25-g mouse has been estimated to range between 1.5 and 2.3 ml (25). To avoid hypotension, surgery was performed with a micro-coagulator and every effort was made to minimize blood losses. Pilot studies, however, showed that opening the chest caused a significant drop in arterial blood pressure so that the mice became severely hypotensive despite the administration of 13.3 ml/kg (~ 0.4 ml) of blood (Fig. 5). Besides causing mortality, severe hypotension could lead to myocardial hypoperfusion and, possibly, induce PC as a result of myocardial ischemia and/or reflex adrenergic activation. We therefore modified our protocol by administering three doses of blood (total of 40 ml/kg or ~ 1.2 ml), as detailed in METHODS, which resulted in values of mean arterial blood pressure >80 mmHg throughout a sequence of six coronary occlusion-reperfusion cycles (Fig. 5).

These values of arterial pressure are within the range reported by others in normal mice (19, 21, 25, 26, 28, 34, 42, 47, 50, 52). Therefore, it is unlikely that the sequence of six cycles of occlusion-reperfusion produced PC because of hypotension-induced ischemia. Such a possibility was further ruled out by the results obtained in sham-preconditioned mice (*groups III, V, and VII*).

Comparison with previous studies

Besides the physiological relevance of the model, the reliability of the measurements of infarct size was felt to be of paramount importance in the outcome of the present investigation. Several modifications of the postmortem perfusion technique were implemented during the development of this protocol, which led to a progressive improvement in tissue staining. The final protocol described in METHODS resulted in excellent staining and clear delineation of both region at risk and infarction, as shown in Figs. 2–4. On the basis of the quality of the staining, we feel that the precision of the measurements of infarct size was at least equal to that previously achieved in our laboratory in dogs (35), pigs (39), and rabbits (2, 38, 48). This conclusion is further corroborated by the small interobserver and intraobserver variabilities in our infarct size measurements, as detailed in RESULTS. These considerations indicate that the results obtained in this murine model are accurate and reproducible.

With the use of this model, the average infarct size in nonpreconditioned mice (*groups I–III, V, and VII* combined) was found to be 52% of the region at risk, which is similar to the average infarct size measured after the same duration of coronary occlusion (30 min) in conscious rabbits [$56.9 \pm 5.9\%$ (Ref. 38) and $56.8 \pm 5.3\%$ (Ref. 48) of the region at risk] and in open-chest rabbits [$52.0 \pm 5.2\%$ (Ref. 30), $53.6 \pm 5.7\%$ (Ref. 5), $48.1 \pm 3.9\%$ (Ref. 4), and $49.1 \pm 4.3\%$ (Ref. 3) of the region at risk]. The ranges of individual infarct sizes (Fig. 6) and the slopes and *x*-intercepts of the infarct size-risk region relationships (Fig. 7) were also similar to those previously observed in conscious rabbits after a 30-min occlusion (38, 48). The average infarct size measured in nonpreconditioned mice in this study (52% of the risk region) is larger than that reported by Michael et al. (32) in nonpreconditioned mice subjected to 30 min of occlusion and 24 h of reperfusion ($34.4 \pm 9.2\%$) and by Hutter et al. (22) in six nonpreconditioned mice subjected to 30 min of occlusion and 2 h of reperfusion ($33.4 \pm 4.5\%$). The reason(s) for the differences between these previous studies (22, 32) and the present results is unknown. Differences in body temperature might be a factor. Because in these investigations (22, 32) the heart rate was not reported and arterial blood pressure, arterial pH, and arterial P_{O_2} were not measured, it is not possible to compare these variables with the heart rate, arterial pressure, arterial pH, and arterial P_{O_2} in our study.

Early and late PC in mice

Although the early phase of ischemic PC has been consistently observed in every species studied heretofore (1, 13, 16), controversy persists as to whether late PC is a universal or a species-specific phenomenon, since this phase of ischemic PC has been reported in canines (27) and rabbits (3–5, 30, 38, 48, 55) but not in pigs (39) or rats (24). The present results clearly demonstrate that a robust PC effect can be induced in mice during both the early and the late phases of protection. The magnitude of the infarct-sparing effect afforded by early PC (~75% reduction in average infarct size) was impressive. The protection afforded by the late phase of PC was also quite powerful (~48–55% reduction in average infarct size) (Fig. 6). The magnitude of the early and late infarct-sparing effects was roughly comparable to that reported in most previous studies in larger species (1, 5, 11, 13, 14, 16, 27, 30, 31, 33, 38, 39, 48). The design of the present investigation enabled us to perform a direct comparison between the relative potencies of the early and the late phase of ischemic PC, because the same experimental conditions and techniques and the same ischemic PC

protocol (six cycles of 4-min occlusion and 4-min reperfusion) were used to study both phases. As shown in Fig. 6, the average reduction in infarct size afforded by early PC in *group IV* was greater than that afforded by late PC in *group VIII*; also, the spread of the data around the mean was less in *group IV* than in *group VIII*, indicating more consistent protection (Fig. 6). It therefore appears that, in the mouse, the infarct-sparing effects of early PC are more powerful than those of late PC, which is consistent with the observations made in other species (1, 3–5, 11, 13–16, 23, 27, 30, 31, 33, 38, 39, 48, 53, 55, 56).

To rule out the possibility that the PC effects could have been induced by the surgical procedure rather than by ischemia, we studied three groups of sham-preconditioned animals: *group III* (early PC sham), in which the chest was left open for 60 min immediately before the 30-min coronary occlusion, and *groups V* (late PC sham, 4-h reperfusion) and *VII* (late PC sham, 24-h reperfusion), in which the chest was left open for 60 min 24 h before the 30-min occlusion. In these groups, a suture was placed under the coronary artery and, in *groups V* and *VII*, left in place for 24 h, just as in the preconditioned groups. The fact that infarct size in *groups III*, *V*, and *VII* was indistinguishable from that in control mice that were not subjected to the 60-min open-chest state (*groups I* and *II*) (Fig. 6) demonstrates that neither the stress of surgery nor the placement of the suture was sufficient to elicit a PC effect.

In view of the added complexity inherent in following mice for 24 h after reperfusion, we investigated whether extending the reflow period beyond 4 h was important for assessing the final extent of infarct size. Birnbaum et al. (7) have reported that at least 3 h of reperfusion are necessary to assess the final extent of infarction in rabbits. Accordingly, we allowed a minimum of 4 h of reperfusion. When infarct size was compared after 4 and 24 h of reperfusion, the results were similar in both nonpreconditioned (*group II* vs. *group I*; *group VII* vs. *group V*) and preconditioned hearts (*group VIII* vs. *group VI*) (Fig. 6), supporting the conclusion that a 4-h reperfusion interval is sufficient to evaluate ischemic PC in the mouse. This information should be useful in designing future studies, particularly studies of early PC, which could be done acutely without the need for survival surgery.

In conclusion, with the development of genetically engineered mice, there is increasing interest in the use of transgenic or knockout mice as a tool to interrogate the cellular mechanisms of cardiovascular disease. Through overexpression or targeted disruption of specific genes, these murine models provide a unique approach to understanding the role of specific gene products in abnormal cardiovascular function. In the case of ischemic PC, however, the exploitation of genetic manipulations has been hindered by the lack of in vivo physiological correlates. The present study describes a new mouse model of both early and late ischemic PC, in which several fundamental physiological variables are carefully controlled and kept within normal limits. Mortality is relatively low (<20%). Measurements of infarct size are accurate and reproducible. Our results demonstrate that a robust infarct-sparing effect occurs during the early and the late phases of PC in the mouse and that the quantitative aspects of this effect are consistent with prior experience in other species. This murine model should be useful for elucidating the cellular mechanisms of ischemic PC by making it possible to apply molecular biology techniques to intact animal preparations to dissect the precise roles of individual proteins.

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Fig. 1.

Experimental protocol. Eight groups of mice were studied. Mice in *group I* [control group, 4-h reperfusion (R); $n = 9$] underwent a 30-min coronary occlusion (O) followed by 4 h of reperfusion. Mice in *group II* (control group, 24-h R; $n = 14$) underwent a 30-min coronary occlusion followed by 24 h of reperfusion. In *group III* (early PC sham group; $n = 11$), the chest was opened for 1 h before a 30-min coronary occlusion followed by 24 h of reperfusion (the 1 h of open-chest state corresponded to the time interval necessary to perform 6 cycles of occlusion-reperfusion in *group IV*). Mice in *group IV* (early PC group; $n = 12$) were preconditioned with a sequence of 6 cycles of 4-min occlusion and 4-min reperfusion; 10 min later, they underwent a 30-min coronary occlusion followed by 24 h of reperfusion. Mice in *groups V* (late PC sham group, 4-h R; $n = 13$) and *VII* (late PC sham group, 24-h R; $n = 6$) underwent a thoracotomy and 1 h of open-chest state (without coronary occlusion) on *day 1* (the 1 h of open-chest state corresponded to the time interval necessary to perform 6 occlusion-reperfusion cycles in *groups VI* and *VIII*); 24 h later (*day 2*), they underwent a 30-min coronary occlusion followed by 4 (*group V*) or 24 h (*group VII*) of reperfusion. Mice in *groups VI* (late PC group, 4-h R; $n = 14$) and *VIII* (late PC group, 24-h R; $n = 13$) were preconditioned with a sequence of 6 cycles of 4-min occlusion and 4-min reperfusion on *day 1*; on *day 2*, they were subjected to a 30-min coronary occlusion followed by 4 (*group VI*) or 24 h (*group VIII*) of reperfusion.

Fig. 2.

Representative example of a heart from *group II* (control group subjected to a 30-min coronary occlusion and 24 h of reperfusion). The infarcted region was delineated by perfusing the aortic root with 2,3,5-triphenyltetrazolium chloride; the region at risk was delineated by perfusing the aortic root with phthalo blue after tying the previously occluded artery (see text for details). As a result of this procedure, the nonischemic portion of the left ventricle (LV) was stained dark blue and viable tissue within the region at risk was stained bright red, whereas infarcted tissue was light yellow. Note the large, confluent areas of infarction spanning most of the thickness of the LV wall, with thin rims of viable subendocardial tissue. This pattern was characteristic of all 5 nonpreconditioned groups (*groups I–III, V, and VII*). Scale at *bottom* is in mm.

Fig. 3. Representative example of a heart from *group IV* (early PC group, 24-h reperfusion) subjected to a sequence of 6 cycles of 4-min occlusion and 4-min reperfusion followed, 10 min later, by a 30-min occlusion and 24 h of reperfusion. Postmortem perfusion was performed as described in Fig. 2. In contrast to the confluent infarction shown in Fig. 2, the 30-min coronary occlusion in this heart resulted in small, sporadic areas of infarction, a pattern that was characteristic of the entire *group IV*, indicating that the sequence of six 4-min occlusions resulted in a powerful early PC effect against infarction. Scale at *bottom* is in mm.

Fig. 4.

Representative example of a heart from *group VIII* (late PC, 24-h reperfusion) subjected to a sequence of 6 cycles of 4-min occlusion and 4-min reperfusion followed, 24 h later, by a 30-min occlusion and 24 h of reperfusion. Postmortem perfusion was performed as described in Fig. 2. In contrast to the confluent infarction shown in Fig. 2, the 30-min occlusion in this heart resulted in patchy areas of infarction, a pattern that was characteristic of both *groups VI* and *VIII*, indicating that the sequence of six 4-min occlusions induced a powerful late PC effect against infarction. Scale at *bottom* is in mm.

Fig. 5.

Pentobarbital-anesthetized mice underwent open-chest surgery. Mean arterial blood pressure was measured by cannulating the carotid artery. Different protocols for fluid replacement were tested. In the 1st group of mice (○, $n = 5$), only 1 dose of 13.3 ml/kg blood (~0.4 ml) was given (immediately after the chest was opened). In these mice, mean arterial pressure fell to 63.4 ± 3.7 mmHg after the chest was opened (probably due to the loss of negative intrathoracic pressure and to positive end-expiratory pressure). After the chest was closed, blood pressure fell again to a nadir of 63.0 ± 9.7 mmHg. Thus 1 dose of 13.3 ml/kg blood was insufficient to maintain a stable arterial pressure. In the 2nd group of mice (●, $n = 3$), 3 doses of blood were given: the 1st before the chest was opened, the 2nd immediately after the chest was opened, and the 3rd after the chest was closed. Each dose consisted of 13.3 ml/kg (~0.4 ml). With this protocol, mean arterial blood pressure remained 80 mmHg throughout the experiment. A 3rd group of mice (▲, ischemic PC group; $n = 4$) was subjected to a sequence of 6 cycles of 4-min coronary occlusion and 4-min reperfusion and was given 3 doses of blood according to the protocol for the 2nd group. In the 3rd group, mean arterial pressure was also maintained 80 mmHg throughout experiment. Consequently, the 3-dose protocol was used for present studies. vent, Ventilator (time point when mice were connected to ventilator).

Fig. 6. Myocardial infarct size in *groups I–VIII*. Infarct size is expressed as a percentage of region at risk of infarction. ○, Individual mice; ●, mean ± SE for respective group.

Fig. 7.

Relationship between size of region at risk and size of myocardial infarction. Graphs show individual values and regression lines obtained by linear regression analysis for the various groups. *Left:* control groups, *groups I and II*. *Middle:* studies of early PC, *groups III and IV*. *Right:* studies of late PC, *groups V–VIII*. In all groups, infarct size was positively and linearly related to risk region size. Linear regression equations were as follows: *group I*, $y = -2.55 + 0.57x$, $r = 0.87$, $P < 0.005$; *group II*, $y = -2.3 + 0.59x$, $r = 0.77$, $P < 0.001$; *group III*, $y = -1.29 + 0.59x$, $r = 0.85$, $P < 0.001$; *group IV*, $y = -8.34 + 0.31x$, $r = 0.77$, $P < 0.005$; *group V*, $y = -9.11 + 0.71x$, $r = 0.87$, $P < 0.001$; *group VI*, $y = -0.41 + 0.23x$, $r = 0.64$, $P < 0.05$; *group VII*, $y = -1.05 + 0.53x$, $r = 0.91$, $P = 0.01$; and *group VIII*, $y = 9.01 + 0.05x$, $r = 0.16$, $P = 0.60$. Analysis of covariance demonstrated that the regression line for *group IV* was significantly different from that for *group III* and that the regression lines for *groups VI and VIII* were significantly different from those for *groups V and VII*, respectively ($P < 0.05$ for each comparison), indicating that, for any given risk region size, infarct size was smaller in preconditioned compared with control mice.

Table 1

Arterial blood gases in open-chest mice

	<i>Group A</i>	<i>Group B</i>	<i>Group C</i>
<i>n</i>	6	6	11
Ventilation rate, breaths/min	95±1	105±2	121±1
Tidal volume, ml	2.2±0.1	2.2±0.1	2.2±0.1
PaO ₂ , mmHg	176.6±63.9	327.2±45.2	321.0±36.1
pH	7.30±0.02	7.39±0.01	7.51±0.02
PaCO ₂ , mmHg	43.3±2.4	31.0±2.3	17.8±2.3
HCO ₃ ⁻ , mM	21.6±1.5	18.6±1.6	14.1±1.5

Data are means ± SE. Open-chest mice ($n=23$) were ventilated using a tidal volume of 2.2 ± 0.1 ml at different rates to identify the optimal ventilation rate. Mice were allowed to stabilize for 40 min after the chest was opened, and then arterial blood gases were measured in each animal. In mice ventilated at a rate of 95 ± 1 breaths/min (*Group A*; $n=6$), arterial pH was 7.30 ± 0.02 . Increasing ventilation rate to 105 ± 2 breaths/min (*Group B*; $n=6$) resulted in a normal arterial pH (7.39 ± 0.01) as well as a higher arterial PO₂ (PaO₂; 327 ± 45 mmHg). A further increase in ventilation rate to 121 ± 1 breaths/min (*Group C*; $n=11$) caused arterial pH to be abnormally high (7.51 ± 0.02) without any further increase in PaO₂ (321 ± 36 mmHg). Accordingly, a ventilation rate of 105 breaths/min was used in the present study. PaCO₂, arterial PCO₂.

Table 2

Rectal temperature and heart rate on the day of the 30-min coronary occlusion

	Preocclusion	Occlusion		Reperfusion		
		5 min	30 min	5 min	15 min	60 min
		Temperature, °C				
Day 1						
Group I	37.1±0.1	37.0±0.2	37.6±0.1	37.5±0.3	36.8±0.2	36.5±0.1
Group II	37.0±0.2	37.4±0.1	36.9±0.1	37.1±0.1	36.8±0.1	36.8±0.1
Group III	37.0±0.1	37.1±0.2	36.8±0.1	36.8±0.2	36.8±0.2	36.8±0.2
Group IV	37.3±0.2	37.0±0.1	37.2±0.1	37.1±0.1	36.7±0.1	36.7±0.1
Day 2						
Group V	36.6±0.2	37.2±0.1	37.2±0.1	37.3±0.1	37.2±0.1	37.2±0.1
Group VI	36.4±0.4	37.3±0.1	37.4±0.1	37.1±0.1	37.0±0.2	37.2±0.1
Group VII	37.2±0.1	37.2±0.2	37.0±0.2	36.9±0.1	36.9±0.1	37.0±0.2
Group VIII	37.0±0.1	37.1±0.1	37.0±0.1	37.0±0.1	36.9±0.1	36.9±0.1
Heart rate, beats/min						
Day 1						
Group I	555±16	533±11	528±13	550±14	558±14	578±19
Group II	545±14	585±17	593±19	580±19	566±14	586±13
Group III	561±18	588±28	555±26	556±25	538±26	575±28
Group IV	549±20	551±23	543±18	536±16	532±15	562±20
Day 2						
Group V	601±18 [*]	626±29 [*]	609±32 [*]	616±26 [*]	630±29 [*]	649±28 [*]
Group VI	600±10 [*]	636±9 [*]	597±15 [*]	580±10	576±11	596±13
Group VII	584±16	635±17 [†]	639±23 [†]	641±30 [†]	626±32 [†]	654±33 [†]
Group VIII	634±36 [†]	678±21 [†]	687±14 [†]	673±14 [†]	669±13 [†]	677±17 [†]

Data are means ± SE. Measurements of rectal temperature and heart rate were taken before the 30-min coronary occlusion (preocclusion), at 5 and 30 min into the 30-min occlusion, and at 5, 15, and 60 min after reperfusion. Rectal temperature was continuously monitored and carefully controlled throughout the experiment, as detailed in the text. The experimental protocols for the 8 groups of mice are specified in METHODS. Note that heart rate on *day 2* (groups *V*, *VI*, *VII*, and *VIII*) was higher than on *day 1* (groups *I*, *II*, *III*, and *IV*), possibly reflecting the effect of surgical trauma 24 h earlier.

^{*} $P < 0.05$ vs. group *I*.

[†] $P < 0.05$ vs. group *II*.

Table 3

Size of left ventricle, risk region, and infarct

Group	Heart Wt, mg	LV Wt, mg	Risk Region Wt, mg	Infarct Wt, mg	Risk Region, % of LV	Infarct, % of Risk Region	Infarct, % of LV
<i>I</i>	209.4±6.1	148.4±5.6	46.9±4.1	24.0±2.7	31.6±2.7	50.9±2.6	16.0±1.5
<i>II</i>	153.7±4.9	110.9±4.2	45.3±3.3	24.2±2.5	41.0±2.9	53.2±3.6	22.1±2.4
<i>III</i>	159.5±9.4	116.5±7.9	50.4±4.0	28.5±2.8	43.8±3.1	56.7±2.4	24.6±2.1
<i>IV</i>	144.5±4.1	105.7±2.6	51.4±3.7	7.7±1.5 ^{a,b}	49.3±4.0	14.2±1.9 ^{a,b}	7.4±1.5 ^{a,b}
<i>V</i>	195.1±6.3	130.6±4.3	46.3±4.0	23.8±3.3	35.8±3.0	49.4±3.4	18.0±2.3
<i>VI</i>	192.7±8.7	132.9±4.7	49.9±5.3	11.1±1.9 ^{c,d}	38.4±4.2	22.2±2.6 ^{c,d}	8.7±1.6 ^{c,d}
<i>VII</i>	141.5±7.2	101.4±5.8	40.0±5.7	20.2±3.3	39.3±4.7	49.9±4.0	19.9±3.1
<i>VIII</i>	159.6±5.6	115.7±4.1	48.3±4.2	11.5±1.4 ^{b,e}	41.8±3.4	25.9±3.3 ^{b,e}	10.0±1.0 ^{b,e}

Data are means ± SE. Heart wt, total heart weight (ventricles and atria); LV, left ventricle.

^a $p < 0.05$ vs. group III;^b $p < 0.05$ vs. group II;^c $p < 0.05$ vs. group V;^d $p < 0.05$ vs. group I;^e $p < 0.5$ vs. group VII.