Heart Failure

Delayed Postconditioning in the Mouse Heart In Vivo

François Roubille, MD, PhD*; Alicia Franck-Miclo*; Aurélie Covinhes; Chrystel Lafont; Frédéric Cransac, MD; Stéphane Combes, MD; Anne Vincent, PhD; Pierre Fontanaud; Catherine Sportouch-Dukhan, MD; Christelle Redt-Clouet, PhD; Joël Nargeot, PhD; Christophe Piot, MD, PhD; Stéphanie Barrère-Lemaire, PhD

Background—Reperfusion during acute myocardial infarction remains the best treatment for reducing infarct size. Postconditioning, applied at the onset of reperfusion, reduces myocardial infarction both in animals and humans. The objective of this study was to identify the time delay to apply postconditioning at reperfusion, allowing preservation of cardioprotection in the mouse myocardium. This is a major issue in the management of acute myocardial infarction patients.

Methods and Results—Mice were subjected to 40 minutes of ischemia and 60 minutes of reperfusion (IR60). Postconditioning protocols corresponding to repetitive ischemia (3 cycles of 1 minute of ischemia and 1 minute of reperfusion) were applied during early reperfusion at various time durations (Δt) after reopening of the coronary artery (Δt = 10 seconds, 1, 5, 10, 15, 20, 30, and 45 minutes; PostCΔt). Infarct size/area at risk was reduced by 71% in PostCΔt compared with IR60 mice (P = 5 × 10⁻⁶). There was a linear correlation (r² = 0.91) between infarct size and Δt, indicating that the cardioprotective effect of delayed postconditioning was progressively attenuated when Δt time increased. The protective effect of PostCΔt and PostCΔt5 was still effective when the duration of reperfusion was prolonged to 24 hours (IR24 hours; PostCΔt and PostCΔt5 versus IR24 hours, P = 0.001). Similar results were obtained for internucleosomal DNA fragmentation and lactate dehydrogenase release.

Conclusions—This study in our in vivo mouse model of myocardial IR shows for the first time that delaying the intervention of postconditioning to 30 minutes does not abrogate the cardioprotective effect of postconditioning. This finding provides evidence that the time window of protection afforded by postconditioning may be larger than initially reported. (Circulation. 2011;124:1330-1336.)

Key Words: apoptosis ■ ischemia ■ myocardial infarction ■ postconditioning ■ reperfusion injury

Acute myocardial infarction is a major cause of heart failure and death. Rapid reperfusion of the ischemic myocardium, by either thrombolyis or primary percutaneous coronary intervention, remains the best treatment for attenuating myocardial infarction. However, reperfusion itself has the potential to initiate additional lethal injury. This deleterious phenomenon culminates in death of cardiac cells that were viable immediately before myocardial reperfusion.1 New strategies that directly target the reperfusion phase could improve clinical outcomes of acute myocardial infarction.2-4 One such strategy, first described by Zhao and colleagues,5 is called postconditioning. Applying intermittent episodes of myocardial ischemia/reperfusion at the early moments of reperfusion reduces myocardial infarction in every animal species tested, including mice.6 Similar beneficial effects were recently demonstrated in patients who underwent primary percutaneous coronary intervention for acute coronary occlusion.3,4

Editorial see p 1315
Clinical Perspective on p 1336

There appears to be a consensus that the delay after which the first reocclusion is established can only be short, but the available data are surprisingly sparse.7 In a rat model, the beneficial effect of postconditioning was reported as lost when the delay between reperfusion and the first reocclusion was shifted from 10 to 60 seconds.8 Since then, postconditioning maneuvers were initiated within 1
minute of reperfusion in the majority of studies. To the best of our knowledge, no study has considered delayed postconditioning in mice hearts in vivo. The main objective of the present study was to compare infarct sizes, as assessed by 2,3,5-triphenyltetrazolium chloride (TTC) and DNA fragmentation, in mice subjected to a postconditioning algorithm. The latter was applied after various time intervals of reperfusion (ie, PostC₄₀) in which the duration of the initial reperfusion period immediately after the index ischemia and before the postconditioning maneuvers ranged from 1 to 45 minutes.

Methods
All the experiments were carried out in accordance with the European Communities directive of November 1986 and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (Publication No. 85–23, revised 1996). The surgical protocols were approved by the regional committee for animal research.

Surgical Preparation
Acute myocardial ischemia and reperfusion were performed in C57Bl6 mice as previously described.9 Male mice (22 to 30 g) were anesthetized with an intramuscular injection of an anesthetic mixture comprising ketamine (50 mg/kg), xylazine (10 mg/kg; Rompun 2%), Bayer, France), and chlorpromazine (1.25 mg/kg; Largactil 5 mg/mL, Sanofi-aventis, France). Mice were ventilated via a tracheal intubation on a Harvard rodent respirator (tidal volume, 7.2 mL/kg body mass; respiratory rate, 200 breaths per min). Body temperature was maintained constant between 36.8°C and 37°C by a thermoregulated surgical table connected to a rectal probe. After a second injection of ketamine (50 mg/kg) and xylazine (10 mg/kg), the chest was opened by a left lateral thoracotomy, and a reversible coronary artery snare occluder was placed around the left coronary artery. All animals underwent 40 minutes of ischemia followed by reperfusion by loosening of the knot. For reperfusion time duration ≤90 minutes, a third administration of ketamine/xylazine was performed intramuscularly (same dose as in the previous injection). The heart was removed at the end of the surgical protocol. When reperfusion lasted 24 hours, mice underwent a recovery protocol comprising subcutaneous administration of lidocaine (1.5 mg/kg; Xylocaine 10 mg/mL, AstraZeneca, France) and postoperative awakening in an emergency care unit maintained at 28°C with an oxygen supply and controlled humidity. After a 2-hour period of stabilization, mice were allowed to recover in a ventilated cabinet for 20 hours. Mice subjected to 24 hours of reperfusion were anesthetized again according to the same protocol described for short period of reperfusion, and the heart was removed for infarct size and apoptosis analysis. In sham-operated mice (n=3), the suture was passed but not tied.

Experimental Design
All animals were randomly assigned to the surgical groups. In the first group, IR₄₀, all mice were subjected to 40 minutes of ischemia followed by different durations of reperfusion corresponding to 0, 5, 10, 15, 30, 45, 60, or 90 minutes or 24 hours (IR₄₀; n=6; IR₁₀, n=10; IR₁₅, n=10; IR₃₀, n=10; IR₄₅, n=10; IR₆₀, n=10; IR₉₀, n=10; IR₂₄ₕ, n=32, respectively; Figure 1A). In the second group, all mice were subjected to 40 minutes of ischemia followed by either 60 minutes (IR₂₄ₜ) or a prolonged period of 24 hours (IR₂₄ₜ; n=32) of reperfusion (Figure 1B). A postconditioning protocol (3 cycles of 1 minute of occlusion and 1 minute of reperfusion) was applied in addition to the IR₄₀ (n=22) or IR₂₄ₜ (n=32) protocol after various Δt times of reperfusion with Δt=10 seconds or 1, 5, 10, 15, 30, or 45 minutes in postconditioned animals (PostC₄₀; PostC₉₀, n=10; PostC₄₅, n=10; PostC₆₀, n=10; PostC₉₀, n=10; PostC₂₄ₜ, n=32; Figure 1B).

Infarct Size Assessment
Hearts were dissected out, and the LVs were sliced transversally into 1-mm-thick sections and incubated in a 1% solution of TTC (Sigma Aldrich) for 15 minutes at 37°C. After fixation in a 4% paraformaldehyde-PBS solution, the slices were weighted and each side was photographed with an Olympus camera. The ischemic risk area (area at risk [AR], unstained in blue) and the infarcted area (unstained by TTC) were measured by planimetry with Image J (Scion Corp, Frederick, MD). Infarct size was expressed as a percentage of the ischemic risk area.

DNA Fragmentation Assay
Specific DNA fragmentation was quantified in transmural samples of nonischemic or ischemic areas of the LVs with an ELISA kit (Roche Diagnostics), as previously described.

Lactate Dehydrogenase Serum Concentration Determination
Blood sampling and serum analysis were carried out at 16 hours of reperfusion corresponding to the lactate dehydrogenase peak release. Lactate dehydrogenase concentrations were measured with a colorimetric test according to the manufacturer’s instructions (MaxDiscovery Lactate Dehydrogenase Enzymatic Assay kit, B100 Scientific Corp).

Statistical Analysis
All values are expressed as mean±SD. Multiple comparisons between groups were assessed by the Kruskal-Wallis nonparametric test followed by the Dunn post hoc test when appropriate. Values of P<0.05 were accepted as statistically significant. The P values, indicated in the text, were noted in the figures as P=NS for P>0.05. Data were analyzed with GraphPad Prism (GraphPad Software, San Diego, CA) and Statistica (Statsoft, Inc, Tulsa, OK).

Figure 1. Experimental protocols. C57Bl6 mice underwent a surgical protocol of myocardial ischemia/reperfusion (IR). The black box represents the period of ischemia. Infarct size or cell death measurements were performed at the end of surgery for each protocol (indicated by □ ). A, All mice were subjected to 40 minutes of ischemia followed by different time durations (Δt) of reperfusion corresponding to Δt=0, 5, 10, 15, 30, 45, 60, or 90 minutes or 24 hours. B, Mice were submitted to 40 minutes of ischemia and a period of 60 minutes (IR₆₀) or a prolonged period of 24 hours (IR₂₄ₜ) of reperfusion. A conditioning stimulus comprising 3 cycles of 1 minute of ischemia and 1 minute reperfusion (noted by the 3 black bars) was applied at various Δt times (Δt=10 seconds to 1, 5, 10, 15, 20, 30, or 45 minutes) after the onset of reperfusion (PostC₄₀).

At the end of reperfusion, the artery was reocluded and the phtalocyanine blue dye was injected into the left ventricle (LV) cavity and allowed to perfuse the nonischemic portions of the myocardium. Hearts were harvested and dedicated to infarct size, DNA fragmentation, or lactate dehydrogenase serum concentration measurements.
In Figure 2A, infarct size measurements (IS, expressed as mean±SD) were fitted using the Boltzmann sigmoid function determined by

\[ f(t_{\text{reperf}}) = IS_{\text{min}} + \frac{IS_{\text{max}} - IS_{\text{min}}}{1 + e^{\left(\frac{t - t_{1/2}}{k}\right)}} \]

where \( t_{\text{reperf}} \) is the time duration of reperfusion (in minutes), \( IS_{\text{min}} \) (minimal value of IS) and \( IS_{\text{max}} \) (maximal value of IS) are the asymptotes of the curve, \( t_{1/2} \) is the time at which IS is halfway, and \( k \) is the slope factor.

In Figure 3B, infarct size data (mean±SD) were fitted by a linear regression model with the least-squares method (Graph Pad Prism software) based on the minimization of the sum of squares of the vertical distances of the points from the line.

**Results**

**Effect of Reperfusion Time Duration on Infarct Size in Mice Hearts In Vivo**

Average AR/LV mass and infarct size/AR were determined in all mice subjected to ischemia/reperfusion. Figure 2A shows the kinetics of infarct size assessed by TTC in mice subjected to different durations of reperfusion (ie, IR\(_{24}\)) after 40 minutes of ischemia, ranging from 5 to 90 minutes (IR\(_0\), IR\(_5\), IR\(_10\), IR\(_15\), IR\(_30\), IR\(_45\), IR\(_60\), and IR\(_90\)). There was no statistical difference between AR/LV among groups (P=0.22; Figure 2B).

Infarct size increased progressively with the duration of reperfusion to reach a plateau at 60 minutes of reperfusion (infarct size/AR: 12.7±1.5 for IR\(_0\); IR\(_5\): 12.5±1.6, n=5; IR\(_10\): 12.7±1.6, n=5; IR\(_15\): 15.4±2.7, n=5; IR\(_30\): 33.3±7.6, n=5; IR\(_45\): 43.2±4.1, n=5; IR\(_60\): 50.6±7.6, n=14; IR\(_90\): 47.2±4.7, n=5; Figure 2A). Indeed, there was no statistical difference in infarct size measure between 60 and 90 minutes of reperfusion (P=1). These results indicate that 60 minutes of reperfusion is efficient to assess infarct size with TTC staining in our in vivo mouse model, which indicates that reperfusion injuries occur faster in the mouse heart than in other larger species.

Ratio of soluble nucleosomes was measured in the ischemic versus the nonischemic portion of LV tissues from mice subjected to IR\(_0\), IR\(_5\), IR\(_10\), IR\(_15\), IR\(_30\), IR\(_45\), IR\(_60\), and IR\(_90\) protocols. DNA fragmentation mean values also increase during reperfusion (Figure 2C) and seem to reach a plateau at 60 minutes of reperfusion. Compared with infarct size kinetics (Figure 2A), a slight delay is observed before the rise of the DNA fragmentation mean values.

Data point values for IR\(_{24}\) hours (mean±SD) were plotted on Figure 2A and 2B as an indication, but were not included in the fitting of the curve (Figure 2A) because of both the differences in the anesthetic protocol and the existence of a postoperative recovery phase for IR\(_{24}\) hours.

**Time-Dependent Cardioprotective Effect of Delayed Postconditioning**

To assess the cardioprotective effects of postconditioning, all mice were subjected to 40 minutes of ischemia and 60 minutes of reperfusion (Figure 3). PostC\(_{5}\) and PostC\(_{15}\) hearts showed smaller infarct sizes than control IR\(_{60}\) hearts (P=7×10\(^{-6}\)).

Average infarct size/AR was 71% smaller in the group subjected to the PostC\(_{5}\) protocol than in IR\(_{60}\) animals (14.8±9.3%, n=12, versus 50.6±7.6%, n=14; P=5×10\(^{-6}\)).

This protective effect was also observed when the postconditioning algorithm was applied 10 seconds after the opening of the coronary artery during the very early reperfusion phase (P=10\(^{-5}\); PostC\(_{5}\): 14.8±9.3%, n=12, versus 18.19±5.3%, n=6, for PostC\(_{10}\); P=1.00; and PostC\(_{10}\): 18.19±5.3%,
Figure 3. Effect of delayed postconditioning on infarct size and DNA fragmentation. Infarct size (in percent of area at risk [AR]) and internucleosomal DNA fragmentation determined by ELISA were quantified in mice subjected to IR60 and additional conditioning stimuli applied 1 minute (postconditioning PostC1d) or 15 minutes (PostC15d) after the onset of reperfusion. A, Scatter dot blots for infarct size/AR (left) and AR/left ventricular (LV) mass (right) and mean ± SD were plotted for PostC1d, PostC15d, and IR60. Representative pictures of tissue samples (LV sections obtained after dual blue and 2,3,5-triphenyltetrazolium chloride dye coloration) for each group are reported on top of the graph for each protocol. *P<0.05 and **P<0.001 vs IR60; #P>0.05 vs PostC1d. B, Mean ± SD for infarct size/AR measured in all groups of PostC1d. There was a linear regression (r²=0.91) between infarct size (in percent of AR) and Δt (delay of postconditioning application in minutes), indicating that the cardioprotective effect of delayed postconditioning was progressively attenuated when Δt time between the first reopening of the artery and the application of postconditioning was increased. A dotted line for infarct size mean of IR60 is reported for animals not undergoing postconditioning. *P<0.05, **P<0.001, ***P<0.0001, and #P>0.05 vs IR60. C, The ratio of soluble nucleosomes in the ischemic vs the nonischemic portion (I/NI ratio) of LV tissues was significantly reduced in both PostC1d and PostC15d hearts compared with IR60. Scatter dots and mean ± SD were plotted for data from each group of animals. #P>0.05 vs PostC1d; *P<0.05 and ***P<0.0001 vs IR60.

n=6, versus 50.6±7.6%, n=14, for IR60; P=0.009; Figure I in the online-only Data Supplement).

When the application of the postconditioning stimulus was delayed to 15 minutes (PostC15d) after the onset of reperfusion, average infarct size/AR was also markedly reduced compared with IR60 hearts (22.1±6.8%, n=6, versus 50.6±7.6%, n=14; P=0.02; Figure 3A, left).

Figure 3B shows the kinetics of infarct size assessed by TTC in mice subjected to different postconditioning algorithms (ie, PostCΔt) in which the duration of the initial reperfusion period immediately after the index ischemia and before the postconditioning maneuver ranged from 1 to 45 minutes (PostCΔt1, PostCΔt5, PostCΔt10, PostCΔt15, PostCΔt30, or PostCΔt45).

There was a linear correlation between infarct size and time delay to postconditioning application (r²=0.91; Figure 3B). The cardioprotective effect of postconditioning was efficient at 30 minutes and statistically lost at 45 minutes (PostCΔt30: 26.1±5.9, n=11, versus IR60: 50.6±7.6, n=14; P=0.004; and PostCΔt45: 32±10.6, n=13, versus IR60: 50.6±7.6, n=14; P=0.36). The AR/LV mass ratio was unchanged among groups (P=0.57; Figure 3A, right).

DNA fragmentation was then measured in mice heart to assess apoptotic cell death. As shown in Figure 3C, the ratio of soluble nucleosome in the ischemic versus the nonischemic portion of LV tissues was significantly reduced in both PostC1d and PostC15d hearts compared with IR60 (P=0.0007; IR60: 5.0±1.7, n=8, versus PostC1d: 1.1±0.5, n=5; P=0.0006; or IR60 vs PostC15d: 2.3±1.2, n=9; P=0.046).

Cardioprotection Induced by Delayed Postconditioning Persists for 24 Hours

To confirm that prolonged reperfusion did not modify our results, the duration of reperfusion was extended to 24 hours in mice subjected to 40 minutes of ischemia. As reported in Figure 4A, infarct size measured by TTC staining was significantly reduced in PostC1d and PostC15d hearts compared with IR24 (P=0.001; ischemic size/AR: IR24 hours, 32.9±6, n=14, versus PostC1d: 18.1±4.8, n=7 [P=0.003] and versus PostC15d: 20.4±4.7, n=6 [P=0.03]). The AR/LV mass ratio was unchanged among groups (P=0.63).

Ischemic-to-nonischemic ratio of LV soluble nucleosomes was significantly reduced on both PostC1d and PostC15d compared with IR24 hours control hearts (P=0.002; IR24 hours: 4.0±2.1, n=9, versus PostC1d: 1.6±0.5, n=7; P=0.043) and with PostC15d (1.3±0.3, n=5; P=0.004; Figure 4B). Similar results were obtained for lactate dehydrogenase release, another marker of myocardial cell death (P=0.001; IR24 hours: 5239±2446 UI/L, n=9, versus PostC1d: 1349±1131 UI/L, n=7; P=0.0013; or versus PostCΔt15: 2248±1165 UI/L; n=8; P=0.03; Figure 4C).

Discussion

In the present study, we tested the hypothesis that the time window of cardioprotection offered by postconditioning in mice may be larger than initially reported. Our results show that postconditioning when applied at 10 seconds or 1, 5, 10, 15, or 30 minutes after the onset of reperfusion reduced infarct size and DNA fragmentation. However, the degree of cardioprotection was conversely proportional to the delay of postconditioning application. Interestingly, cardioprotection afforded by postconditioning at the various delay times persisted for 24 hours.

Application of the postconditioning stimulus was able to prevent the lethal reperfusion injuries taking place during the period consecutive to postconditioning maneuvers. These findings suggest that lethal reperfusion is an ongoing process.
that continues at least 30 minutes after the onset of reperfusion in the mouse heart in vivo. This may have major implications in clinical practice.

**Postconditioning in the Mouse Heart**

In this study, we first confirmed, using phthalocyanine blue dye and TTC, that postconditioning reduces infarct size in the mouse heart, as reported initially by Heusch et al.\(^1\) Furthermore, DNA fragmentation analysis evidenced a postconditioning-induced decrease in apoptosis in the ischemic part of the LV. When applied at the onset of reperfusion, postconditioning was associated with a drastic reduction in infarct size at 60 minutes (≈70%) compared with animals without postconditioning taken as controls (IR\(_{60}\)). The amplitude of infarct size reduction observed is consistent with values reported in different animal models, including mice, ranging from 25% to 70%.\(^5,12,13\)

In terms of the postconditioning efficiency in mice, cardioprotection is dependent on the duration of the ischemia/reperfusion stimulus and on the conditioning algorithm used. There are numerous possible combinations of postconditioning algorithms and their efficiency in different species as reported in the literature (for a review, see the work by Skyschally et al\(^\#\)). The durations of alternating periods of reperfusion and ischemia are empirically shorter in small animals and longer in larger species. In the majority of studies, infarct size reduction in mice was observed only with a 30-minute index ischemia and a shorter duration of ischemia/reperfusion per cycle (ie, 3 to 6 cycles of 10 seconds of reocclusion separated by 10 seconds of reperfusion). However, no data in the literature demonstrate that the mouse heart should be postconditioned only by short periods of 5 or 10 seconds of ischemia/reperfusion.

In our study, we used an index ischemia of 40 minutes and a postconditioning maneuver of 3 cycles of 1 minute of reocclusion separated by 1 minute of reperfusion. The postconditioning protocol is derived from clinical studies, and its efficiency has been proven in humans.\(^3,4\) This particular algorithm used in human trials is similarly efficient in mice in terms of the strong reduction in infarct size.

Moreover, this protocol of 3 cycles of 1 minute of ischemia and 1 minute of reperfusion was realized with a specific silicon snare occluder allowing the full restoration of blood flow and then completion of each reperfusion phase of the algorithm (Figure II and Video I in the online-only Data Supplement).

**Time Window of Protection Afforded by Postconditioning**

Although available data are surprisingly sparse, there appears to be a consensus that the first reocclusion should be operated shortly after ischemia, and that the time window of protection is quite narrow. It was reported in rat hearts in vivo that cardioprotection does not occur if the brief episodes of ischemia/reperfusion that trigger postconditioning are performed after 1 minute of reperfusion.\(^8,15,16\)

This information suggested that the main part of lethal reperfusion injury takes place within the first minute of reflow after a prolonged ischemia. However, several studies in mice, rabbits, dogs, and humans provided evidence that postconditioning still decreased infarct size when the delay of the first reocclusion ranged between 1 and 2 minutes.\(^3,4,12,15,16\) Using our in vivo mouse model of ischemia/reperfusion, we show here that postconditioning intervention applied during the 30 minutes after reperfusion allows us to observe a time-dependent cardioprotective effect, suggesting that the time window of protection afforded by postconditioning is larger than initially reported. Using a shorter postconditioning protocol as in other studies (3 cycles of 10 seconds of ischemia and 10 seconds of reperfusion) in our model of myocardial infarction, we also observed the existence of the time window of cardioprotection in the mouse heart, suggesting that delayed cardioprotection is independent of the postconditioning algorithm used (Figure III in the online-only Data Supplement).
The exact molecular signaling underlying delayed cardioprotection remains unknown. However, different survival pathways, including reperfusion injury salvage kinase (RISK) and survivor activating factor enhancement (SAFE), have been evidenced in the cardioprotective effects of postconditioning. We speculate that these survival pathways may be also involved in delayed postconditioning. Future work is needed to investigate this hypothesis.

Wave Front of Reperfusion Injury
During myocardial reperfusion, the ischemic myocardium is subjected to several abrupt biochemical and metabolic changes. Although it was expected that most lethal events occur during early reperfusion, the dynamic wave front of reperfusion-induced cell death remained largely unexplored. In this study, we show that both infarct size and DNA fragmentation occurs between the increase in infarct size and DNA fragmentation amount (a direct measure of cell death), suggesting that apoptosis also progressively increases over time after a prolonged period of ischemia. One established interpretation is that reperfusion leads to washout of nicotinamide adenine dinucleotide from irreversibly damaged areas. The other, and more challenging, interpretation is that myocardial cell death progresses over time (wave front of reperfusion injury). Consistent with the hypothesis of a wave front phenomenon of reperfusion injury, we observed a similar rise of the DNA fragmentation amount (a direct measure of cell death), suggesting that apoptosis also progressively increases over time after the onset of reflow. However, a shift over time may be noted between the increase in infarct size and DNA fragmentation curves, with DNA fragmentation occurring with some delay at reperfusion before reaching a steady-state value at 60 minutes of reperfusion. This may reflect a degree of necrosis before apoptosis, as already suggested. In this context, delayed postconditioning applied up to 30 minutes after the onset of reperfusion can prevent apoptotic cell death, which drastically increases between 30 and 60 minutes of reperfusion, as indicated in Figure 3B. Postconditioning can fully prevent reperfusion injuries when applied at the onset of reperfusion, whereas delayed postconditioning provides a time-dependent degree of protection. Similar signaling mechanisms are probably involved in both postconditioning and delayed postconditioning. At present, the reasons for the relatively decreased efficiency of delayed postconditioning compared with postconditioning need further investigation. A likely explanation is that delayed postconditioning cannot reverse the apoptosis phenomena that were previously triggered by early reperfusion.

Together, these data support the concept of reperfusion injury but do not completely rule out the possibility of artifacts in the measure of infarct size after short periods of reperfusion owing to experimental conditions.

Acknowledgments
We wish to thank the IFR3 mouse facility. We are grateful to Dominique Haddou and Frédéric Gallardo for their excellent technical assistance concerning animal care.


**CLINICAL PERSPECTIVE**

The major finding of this study is that delayed postconditioning allows infarct size to be reduced in the mouse heart in vivo. This observation emphasizes the fact that the cardioprotection window for postconditioning is wider than initially reported. Such information may be of major importance for clinical applications. Manual thrombectomy, in addition to primary percutaneous coronary intervention, has been shown to improve microvascular reperfusion, with a lower mortality at 1-year follow-up. Unfortunately, routine use of mechanical thrombectomy during primary percutaneous coronary intervention increases procedure time and precludes the application of angioplasty postconditioning in the 1 minute of reflow. Confirmation of the existence of a longer cardioprotection window by postconditioning in a range of animal myocardial infarction models, including larger animals, may justify new clinical trials combining manual thrombectomy and angioplasty postconditioning in patients with acute myocardial infarction in accordance with the recently published recommendations for investigating novel cardioprotective strategies. In addition, demonstration of delayed postconditioning may also be of interest in patients with incomplete reperfusion at hospital admission in whom episodes of brief ischemia/reperfusion may be performed during the percutaneous coronary intervention to further reduce infarct size. However, the translation of cardioprotection from the experiment to the clinic is not obvious, because species differences, age, comorbidities, cotreatments, and the status of the coronary circulation may interfere with postconditioning. We think that our data, which demonstrate the existence of a longer cardioprotection window, are conceptually relevant for a clinical application and make the case for a pharmacological strategy.
Delayed Postconditioning in the Mouse Heart In Vivo
François Roubille, Alicia Franck-Miclo, Aurélie Covinhes, Chrystel Lafont, Frédéric Cransac, Stéphane Combes, Anne Vincent, Pierre Fontanaud, Catherine Sportouch-Dukhan, Christelle Redt-Clouet, Joël Nargeot, Christophe Piot and Stéphanie Barrère-Lemaire

_Circulation_. 2011;124:1330-1336; originally published online August 29, 2011;
doi: 10.1161/CIRCULATIONAHA.111.031864
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/124/12/1330

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2011/08/29/CIRCULATIONAHA.111.031864.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

Delayed postconditioning in the mouse heart in vivo

by

Roubille François, Franck-Miclo Alicia, Covinhes Aurélie, Lafont Chrystel, Cransac Frédéric, Combes Stéphane, Vincent Anne, Fontanaud Pierre, Sportouch-Dukhan Catherine, Redt-Clouet Christelle, Nargeot Joël, Piot Christophe and Barrère-Lemaire Stéphanie.
Legends for Supplemental Figures

Supplemental Figure 1. Influence of a shorter delay time for postconditioning application.

(A) Mice were subjected to 40 minutes of ischemia and 60 minutes of reperfusion (IR_{60'}). Postconditioning protocols comprising 3 cycles of 1 min ischemia -1 min reperfusion were applied 1 min after the opening of the coronary artery (PostC_{Δ1}) or a shorter delay (i.e. 10 seconds) PostC_{Δ10"}. At the end of the surgery, left ventricles were harvested and infarct size was evaluated by the TTC method. The arrow indicates the time of heart removal and infarct size analysis. Results were compared with those obtained in mice subjected to IR_{60'}. Scatter dot for all the values measured for Infarct size/AR (B) and for AR/LV mass (C) and means ± SD were plotted for PostC_{Δ1}, PostC_{Δ10"} and IR_{60'}. Area at risk (in % of LV) was unchanged among groups p=0.14 (noted ns). ** and *** represent respectively p<0.001 and p<0.0001 versus IR_{60'} and # represents p>0.05 versus PostC_{Δ1}.

Supplemental Figure 2. Visualization of the left ventricle blood flow during the ischemia-reperfusion phases of the PostC_{Δ1} algorithm.

The surgical procedure was performed under a stereomicroscope coupled to an EM-CCD camera allowing real-time in vivo imaging of the left ventricle during surgery in an anesthetized mouse. An air-transmission 20x magnification objective was fitted on a fluorescence microscope, equipped with a variable light beam excitation and an EM-CCD camera acquisition setup. The mouse was subjected to 40 minutes of ischemia and a PostC_{Δ1} protocol applied 1 minute after releasing the snare-occluder. A jugular venous catheter was inserted for intravenous administration of fluorescently labeled dextrans. 

(A): A bright field image of the open chest section is shown with the snare occluder in the centre. (B): The heart was imaged following the injection of tetramethylrhodamine fluorescent dextran by the jugular vein 30 seconds before releasing the snare occluder. A scheme mapping the different areas of the heart is shown which delineates the ischemic (noted I) and non ischemic (NI) areas of the left ventricle (LV). (C): Experimental protocol indicating the timing of injection of fluorescein isothiocyanate (FITC, green arrow) and tetramethylrhodamine (TMR, red arrow) dextrans during the surgery before the successive phases of reperfusion (R1, R2, R3, R4) following 40 min of ischemia. 

(D): Heart was imaged during the injection of FITC and TMR fluorescent dextrans into the jugular vein 30 seconds before releasing the snare occluder (D, left panel). Representative pictures of the heart corresponding to the first (R1) and last (R4) opening of the artery during the PostC algorithm are presented. Keeping in mind difficulties associated with heart movement during the in vivo experiments, note that the ischemic area (I) becomes fluorescent in R1 or R4 (D, right panel) in less than 2 sec after releasing the snare occluder. The complete sequence can be viewed in the supplementary video (Supplemental file).

Supplemental Figure 3. Study of postconditioning algorithm duration: 3 cycles of 10 seconds ischemia - 10 sec reperfusion.

(A) Mice were subjected to 40 minutes of ischemia and 60 minutes of reperfusion (IR_{60'}). Additional protocols comprising shorter (10 seconds) algorithms of postconditioning (PostC_{10"}) were applied 10 seconds after reperfusion (PostC_{10"Δ10}) or 15 minutes after reperfusion (PostC_{10"Δ15}). At the end of the surgery, left ventricles were harvested and infarct
size was evaluated by the TTC method (the arrow indicates the time of heart removal and infarct size analysis). Results were compared with those obtained in mice subjected to IR$_{60'}$ and postconditioning algorithms comprising 3 cycles of 1 min ischemia - 1 min reperfusion indicated as PostC$_{Δ1}$ and PostC$_{Δ15}$ (described in Figure 1 of the manuscript). Scatter dots for all the values measured for Infarct size/AR (B) and for AR/LV mass (C) and means ± SD were plotted for PostC$_{Δ1}$, PostC$_{Δ15}$, PostC10''$_{Δ10'}$, PostC10''$_{Δ15}$ and IR$_{60'}$. All the PostC protocols significantly decrease infarct size (in % of AR) compared to IR$_{60'}$ (p=2.10$^{-6}$). Note that delayed cardioprotection is independent of the PostC algorithm used (PostC$_{Δ15}$ versus PostC10''$_{Δ15}$: 21.1 ± 4.1, n=9; p=1 and PostC10''$_{Δ15}$: 21.1 ± 4.1, n=9 versus 50.6 % ± 7.6, n=14; p=0.007). Area at risk (in % of LV) was unchanged among groups (p= 0.22). ***, ** represent p<0.0001 and p<0.001 versus IR$_{60'}$ and # represents p>0.05 versus PostC$_{Δ1}$.

**Legend for supplemental video**

The experiments were performed under a stereomicroscope (M2 Discovery Zeiss) coupled to an EM-CCD camera (C9100 Hamamatsu). The blood flow was visualize in the left ventricle subjected to each reperfusion cycle during the application of the postconditioning protocols (Imaging Platform of our Institute, http://ipam.igf.cnrs.fr). We have used dyes coupled to large molecular weight-dextrans: fluorescein isothiocyanate (FITC) and tetramethylrhodamine dextrans (500 and 150 kDa, respectively Sigma Aldrich) that are valuable tools for studying permeability and microcirculation in vivo. The dyes were injected via the jugular vein 30 seconds before reopening of the coronary artery. The mouse was subjected to 40 minutes of ischemia and a PostC$_{Δ1}$. The protocol of dye injection is described in Supplemental Figure 2C.

The video shows selected sequences of images recorded during the application of the PostC algorithm. The time counter (bottom right) indicates the time during the protocol. The video recording starts at 39 minutes and 30 seconds during the ischemia period (lasting 40 minutes, see supplemental Figure 2C).

Keeping in mind difficulties associated with heart movement during the in vivo experiments, note that the ischemic area becomes fluorescent in R1, R2, R3 and R4 phases in less than 2 sec after releasing the snare occluder. Please note that the fluorescent background noise increases during the recording phases: this is related to the accumulation of the fluorescent dyes over time during the procedure.
Supplemental Figure 1
Influence of a shorter time delay of postconditioning application

A

![Diagram showing the timeline of ischemia and reperfusion with different postconditioning applications: IR_{60'}, PostC_{Δ1}, PostC_{Δ10'}, and 3 cycles (1 min I - 1 min R).]

B

![Bar graph showing infarct size (% AR) with comparison among different postconditioning applications: PostC_{Δ1}, PostC_{Δ10'}, and IR_{60'}. The graph indicates a significant difference among the groups, with IR_{60'} showing the least infarct size.]

C

![Bar graph showing ARLV with comparison among different postconditioning applications: PostC_{Δ1}, PostC_{Δ10'}, and IR_{60'}. The graph indicates no significant difference (ns) among the groups.]
Supplemental Figure 2. Visualization of the left ventricle blood flow during the ischemia-reperfusion phases of the PostCΔ1 algorithm.
Supplemental Figure 3
Study of postconditioning algorithm duration: 3 cycles of 10 seconds ischemia - 10 sec reperfusion.