# Postconditioning Inhibits Mitochondrial Permeability Transition

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***Background***—Brief periods of ischemia performed just at the time of reperfusion can reduce infarct size, a phenomenon called "postconditioning." After reflow, opening of the mitochondrial permeability transition pore (mPTP) has been involved in lethal reperfusion injury. We hypothesized that postconditioning may modulate mPTP opening.

***Methods and Results***—Anesthetized open-chest rabbits underwent 30 minutes of ischemia and 4 hours of reperfusion. Control hearts underwent no additional intervention. Postconditioning consisted of 4 episodes of 1 minute of coronary occlusion and 1 minute of reperfusion performed after 1 minute of reflow after the prolonged ischemia. Preconditioning consisted of 5 minutes of ischemia and 5 minutes of reperfusion before the 30-minute ischemia. An additional group of rabbits received 5 mg/kg IV of NIM811, a specific inhibitor of the mPTP, 1 minute before reperfusion. Infarct size was assessed by triphenyltetrazolium staining. Mitochondria were isolated from the risk region myocardium, and $Ca^{2+}$-induced mPTP opening was assessed by use of a potentiometric method. Postconditioning, preconditioning, and NIM811 significantly limited infarct size, which averaged $29\pm4\%$, $18\pm4\%$, and $20\pm4\%$ of the risk region, respectively, versus $61\pm6\%$ in controls ($P\leq0.001$ versus control). The $Ca^{2+}$ load required to open the mPTP averaged $41\pm4$, $47\pm5$, and $67\pm9$ $\mu$mol/L $CaCl_2$ per mg of mitochondrial proteins in postconditioning, preconditioning, and NIM811, respectively, significantly higher than the value of $16\pm4$ $\mu$mol/L per mg in controls ($P\leq0.05$).

***Conclusions***—Postconditioning inhibits opening of the mPTP and provides a powerful antiischemic protection. (***Circulation***. 2005;111:194-197.)

**B**rief episodes of ischemia render the myocardium more resistant to subsequent prolonged ischemia-reperfusion, a phenomenon called "preconditioning."[1] Recently, Zhao et al[2,3] reported that a similar regimen of brief periods of ischemia applied just after, instead of just before, the sustained ischemia was as protective as preconditioning: they named it "postconditioning." The mechanism of this newly described form of cardioprotection remains unknown.

Mitochondrial permeability transition is a key event in cell death after ischemia-reperfusion.[4–6] Opening of the nonspecific mitochondrial permeability transition pore (mPTP) in the inner mitochondrial membrane results in the collapse of the membrane potential ($\Delta\Psi_m$), uncoupling of the respiratory chain, and efflux of cytochrome *c* and other proapoptotic factors that may lead to either apoptosis or necrosis.[7] $Ca^{2+}$ overload and excessive production of reactive oxygen species in the early minutes of reflow trigger opening of the mPTP and are crucial events in reperfusion injury.[8,9] Griffiths and Halestrap[10] demonstrated in the isolated rat heart that the mPTP remains closed throughout ischemia but opens at the time of reperfusion. We therefore investigated whether postconditioning might inhibit mPTP opening and thereby limit infarct size.

## Methods

All animals were treated in compliance with the "Position of the American Heart Association on Research Animal Use," adopted by the American Heart Association on November 11, 1984.

### Experimental Protocol

Male New Zealand White rabbits (2.2 to 2.5 kg) were anesthetized with xylazine (5 mg/kg) and ketamine (50 mg/kg) and ventilated with room air.[11] A cannula was inserted into the right internal jugular vein for administration of drugs and fluids and into the left carotid artery for measurement of blood pressure. Limb lead II of the ECG was used to measure heart rate. Heart rate and blood pressure were monitored continuously on a Gould recorder (Gould Inc). After a left thoracotomy was performed, the heart was exposed and a snare was passed around a marginal branch of the left circumflex coronary artery to induce regional myocardial ischemia. After the surgical procedure, a 15-minute stabilization period was observed. Animals were then randomly assigned to the experimental groups. All animals underwent 30 minutes of ischemia and 4 hours of reperfusion. They
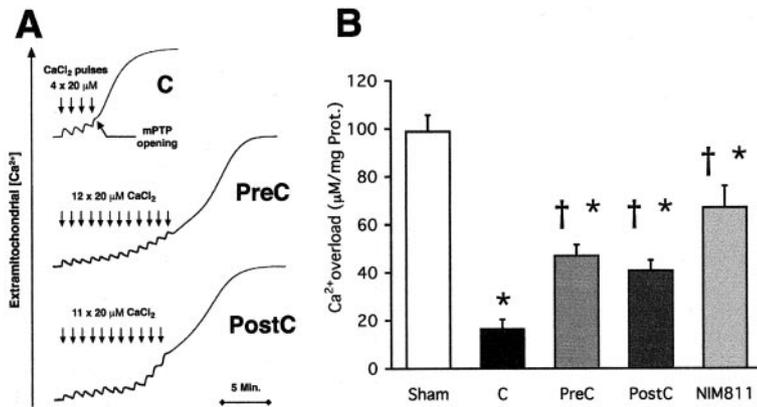
**Figure 1.** Postconditioning delays $Ca^{2+}$-induced mPTP opening. A, Typical example of $Ca^{2+}$-induced mPTP opening recordings in mitochondria isolated from control (C), Pre-C, and Post-C animals. mPTP opening was defined as massive release of $Ca^{2+}$ by mitochondria after progressive $Ca^{2+}$ overload of suspension medium. B, $Ca^{2+}$ required for mPTP opening was significantly reduced vs shams in C group. Post-C, Pre-C, and NIM811 equally inhibited mPTP opening (n=7 to 8 per group). *$P<0.0001$ vs sham; †$P\leq0.01$ vs C.

were randomly allocated to the following groups (n=10 per group): (1) the Control group (C): no other intervention; (2) the Preconditioning group (Pre-C): 1 episode of 5 minutes of ischemia and 5 minutes of reperfusion before the prolonged ischemia; (3) the Postconditioning group (Post-C): no intervention before the 30 minute ischemia. After 1 minute of reflow after the release of the 30-minute occlusion, we performed 4 episodes of 1 minute of ischemia each separated by 1 minute of reperfusion; and (4) the NIM811 group (NIM811): 1 minute before reperfusion, rabbits received an IV bolus injection of 5 mg/kg of NIM811, a nonimmunosuppressive derivative of cyclosporin A that specifically inhibits opening of the mPTP.[12]

An additional subset of rabbits underwent a comparable experimental preparation, but the reperfusion period was extended to 72 hours to determine whether postconditioning simply delays or actually limits irreversible myocardial reperfusion injury. Those animals were randomly assigned to either a control or a Post-C group. They underwent an ischemic insult similar to that in the 4-hour reperfusion protocol, had the chest closed, and were returned to the animal facilities until the end of the reperfusion period (n=9 per group).

### Infarct Size Studies

At the end of the final reperfusion, the coronary artery was briefly reoccluded, and 0.5 mg/kg Uniperse blue pigment (Ciba-Geigy) was injected intravenously to delineate the in vivo area at risk, as previously described.[13] Anesthetized rabbits were then euthanized by an intravenous injection of 4 mEq KCl. The heart was excised and cut into 5 to 6 transverse slices, 2 mm thick, parallel to the atrioventricular groove. Each heart slice was weighed, and its basal surface was photographed for later measurement of the area at risk. After incubation for 15 minutes in a 1% solution of triphenyltetrazolium chloride at 37°C to differentiate infarcted (pale) from viable (brick red) myocardial area, the slices were rephotographed. Enlarged projections of these slices were traced for determination of the boundaries of the area at risk and area of necrosis. The extent of the area at risk and area of necrosis was quantified by computerized planimetry and corrected for the weight of the tissue slices. Determination of area at risk and infarct size was performed in a blinded manner. Total weights of the area at risk and the area of necrosis were then calculated and expressed in grams and as percentage of total left ventricular (LV) weight and of the area at risk weight, respectively. We decided prospectively that hearts with a risk region <10% of the LV weight would be excluded from the study.

### Detection of mPTP Opening in Isolated Mitochondria

Additional animals (n=7 to 8 per group) were used for this in vitro study. At the end of the protocol, myocardium from the area at risk was excised, and mitochondria were isolated, as previously described.[14–16] An additional set of rabbits (Sham, n=10) underwent no intervention for the whole duration of the experiment.

Opening of the mPTP was assessed after in vitro $Ca^{2+}$ overload.[16] Briefly, the isolated mitochondria suspension (5 mg proteins) was placed in a Teflon chamber equipped with a $Ca^{2+}$-selective microelectrode. Modifications of the medium (ie, extramitochondrial) $Ca^{2+}$ concentration were recorded continuously by use of a custommade Synchronie software. At the end of the preincubation period, 20 $\mu$mol/L $CaCl_2$ pulses were performed every 60 seconds. Each 20-$\mu$mol/L $CaCl_2$ pulse is recorded as a peak of extramitochondrial $Ca^{2+}$ concentration (Figure 1). $Ca^{2+}$ is then rapidly taken up by the mitochondria, resulting in a return of extramitochondrial $Ca^{2+}$ concentration to near baseline levels. After sufficient $Ca^{2+}$ loading, extramitochondrial $Ca^{2+}$ concentration increases abruptly, indicating a massive release of $Ca^{2+}$ by mitochondria as a result of mPTP opening (Figure 1A).[16] The amount of $Ca^{2+}$ necessary to trigger this massive $Ca^{2+}$ release is used here as an indicator of the susceptibility of mPTP to $Ca^{2+}$ overload.

### Statistical Analysis

All values are expressed as mean±SEM. Differences in the relationship between infarct size and area at risk were evaluated by ANCOVA and post hoc Tukey's test, with infarct size as the dependent variable and area at risk as the covariate. Hemodynamics were analyzed by use of 2-way ANOVA with repeated measures on one factor.[17] Mitochondrial $Ca^{2+}$ loads required for mPTP opening were analyzed by 1-way ANOVA. Means were compared by the Fisher test. Statistical significance was defined at a value of $P<0.05$.

## Results

### Postconditioning Delays mPTP Opening

The mean amount of $Ca^{2+}$ required to trigger mPTP opening averaged 99±7 $\mu$mol/L $CaCl_2$ per mg mitochondrial proteins in shams (Figure 1B). Ischemia-reperfusion resulted in a significant reduction of $Ca^{2+}$ load necessary to open mPTPs: 16±4 $\mu$mol/L per mg in controls ($P<0.0001$ versus sham). The $Ca^{2+}$ load that induced mPTP opening in Post-C averaged 41±4 $\mu$mol/L per mg, ie, significantly higher than in controls ($P=0.01$ versus control). Pre-C and NIM811 affected mPTP opening to an extent comparable to that in Post-C, with $Ca^{2+}$ load averaging 47±5 and 67±9 $\mu$mol/L per mg, respectively ($P<0.001$ versus control).

### Postconditioning Protects the In Vivo Rabbit Heart

Heart rate and blood pressure were comparable among groups throughout the experiment: blood pressure decreased significantly in all groups after the ischemic insult (Table). Similar pattern of hemodynamics were observed in the 72-hour reperfusion groups, but blood pressure had returned to near

**Heart Rate and Blood Pressure Variations**

| Groups | Baseline | | Pre-CO | | 30-min CO | | 1-Hour Reperfusion | | 4-Hour Reperfusion | |
|---|---|---|---|---|---|---|---|---|---|---|
| | HR, bpm | MBP, mm Hg | HR, bpm | MBP, mm Hg | HR, bpm | MBP, mm Hg | HR, bpm | MBP, mm Hg | HR, bpm | MBP, mm Hg |
| C | 204±10 | 71±5 | 197±15 | 67±6 | 215±10 | 57±4* | 192±15 | 51±5* | 202±15 | 49±4* |
| Pre-C | 173±9 | 70±5 | 172±11 | 62±3 | 194±16 | 55±3* | 168±17 | 61±4 | 206±12 | 53±10* |
| Post-C | 191±10 | 68±2 | 186±11 | 64±3 | 194±7 | 52±3* | 171±8 | 54±2* | 175±6 | 54±3* |
| NIM811 | 191±13 | 74±5 | 195±14 | 71±5 | 202±12 | 50±5* | 201±8 | 56±4* | 196±8 | 50±5* |

HR indicates heart rate; MBP, mean blood pressure; and CO, coronary occlusion. Values are mean±SEM.

*$P<0.05$ vs baseline.

baseline at the end of the experiment. Area at risk averaged 0.93±0.07 g (29±3% of LV weight), 0.91±0.06 g (28±3%), 0.94±0.06 g (37±2%), and 1.11±0.09 g (38±3%) in the C, Pre-C, Post-C, and NIM811 groups, respectively ($P=NS$ among groups). Preconditioning reduced infarct size to 18±4% of area at risk versus 61±6% in controls ($P<0.0001$). Both postconditioning and NIM811 significantly limited infarct size, which averaged 29±4% and 20±4% of the risk region, respectively ($P<0.0001$ versus control, $P=NS$ versus Pre-C) (Figure 2A). For animals with a 72-hour reperfusion period, area at risk averaged 1.00±0.15 g (31±4% of LV weight) and 1.04±0.16 g (35±4%) in C and Post-C, respectively. Infarct size averaged 20±5% of the risk region in the Post-C group versus 48±6% in the C group ($P<0.005$) (Figure 2B). These differences were confirmed by ANCOVA.
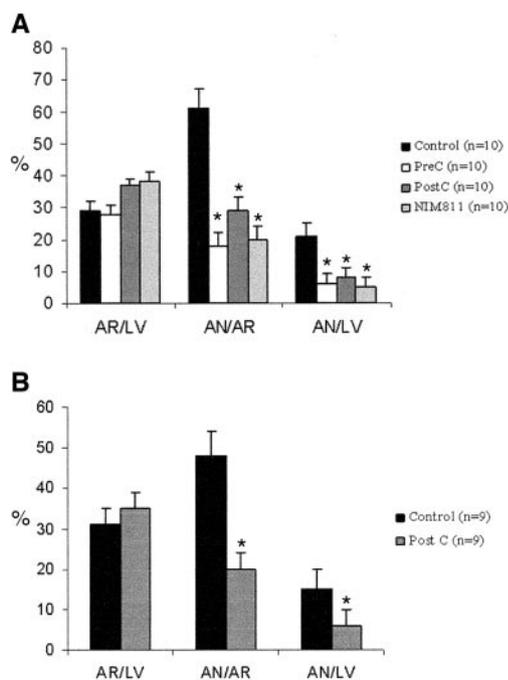


**Figure 2.** Postconditioning reduces infarct size. A, At 4 hours of reperfusion. Area at risk (AR) and area of necrosis (AN) are presented for each group (n=10 per group). Area of necrosis was reduced to a comparable extent in Pre-C, Post-C, and NIM811 groups. *$P<0.05$ vs control. B, At 72 hours of reperfusion. When reperfusion was extended to 72 hours, infarct size–limiting effect of postconditioning was maintained (n=9 per group). *$P<0.05$ vs control.

## Discussion

We report for the first time that postconditioning modifies mPTP opening and reduces infarct size in the rabbit heart.

Zhao et al[2] first described that brief episodes of ischemia performed at the onset of reperfusion after a prolonged ischemia provided a powerful antinecrotic protection. Kin et al[3] proposed that postconditioning might be related to an attenuation of oxygen-derived free radical production in the early minutes of reflow.

The rationale for the present investigation was based on 3 observations. First, the report by Zhao et al indicates that preconditioning and postconditioning are approximately equally protective and that a significant amount of infarct after ischemia-reperfusion is because of reperfusion-induced lethal injury. Second, Griffiths and Halestrap[10] demonstrated that the mPTP remains closed during ischemia but opens in early reperfusion. Third, recent reports demonstrated that mitochondrial permeability transition is involved in preconditioning and that blockade of the mPTP at reflow by cyclosporin A is cardioprotective.[16,18,19] We therefore investigated whether mPTP opening may play a role in postconditioning.

We report that in the in vivo rabbit heart model, postconditioning significantly reduced infarct size and that this protection was preserved after 72 hours of reperfusion, indicating that irreversible myocardial injury was not simply delayed but actually limited. The magnitude of the protective effect of postconditioning was similar to that obtained with NIM811, which specifically inhibited mPTP opening at the time of reperfusion. This is in agreement with previous studies by Hausenloy et al[18] and Javadov et al[19] that demonstrated, using cyclosporin A in the isolated rat heart model, that mPTP is important in myocardial lethal reperfusion injury.

We demonstrated here that mitochondria isolated from postconditioned myocardium display an increased resistance to $Ca^{2+}$ loading. In other words, postconditioning delays $Ca^{2+}$-induced mPTP opening. This pattern of $Ca^{2+}$-induced mPTP opening is very similar to that of preconditioned hearts and mimics those recorded in rabbit hearts treated at the time of reperfusion by the specific mPTP inhibitor NIM811. Although a full demonstration of a causal relation between postconditioning and inhibition of the mPTP will require additional investigations, the present data strongly suggest that mitochondrial permeability transition is an important mediator of this cardioprotection. How postconditioning modulates mPTP opening will need further investigations. On

the basis of the initial studies by Zhao et al and Kin et al, it would be worth determining whether the reduced production of reactive oxygen species after postconditioning may be responsible for delaying mPTP opening.[2,3] One could hypothesize that postconditioning may alter the production of oxygen-derived free radicals by the respiratory chain and thereby delay opening of the mPTP; this, however, remains to be determined. Tsang et al[20] recently reported that postconditioning activates the phosphatidylinositol 3 kinase (PI3K)–Akt pathway and its downstream target, endothelial nitric oxide synthase (eNOS). Yang et al[21] demonstrated that pharmacological inhibition of eNOS and blockade of the mitochondrial $K^+_{ATP}$ channels prevent infarct size limitation by postconditioning. Whether activation of the PI3K-Akt-eNOS cascade may inhibit mPTP opening and be responsible for the protective effect of postconditioning remains to be determined.[22] Recent preconditioning studies also prompt us to investigate matrix $Ca^{2+}$ accumulation and pH variations in the influence of postconditioning on mPTP opening.[23–25]

Postconditioning seems to be a fairly simple strategy to apply during coronary angioplasty in patients with ongoing acute myocardial infarction. It is still unknown, however, whether postconditioning is feasible, safe, and efficient in those patients. Although clear evidence that lethal myocardial reperfusion injury exists in humans is lacking, the potential of postconditioning (ie, limitation of infarct size and improvement in prognosis) must be tested in clinical trials. As an alternative in those patients who cannot undergo coronary angioplasty, pharmacological inhibition of the mPTP at the time of reperfusion may be of major interest as an adjunct therapy to thrombolysis. Clinical investigations are needed to determine the potential benefit of the inhibition of mPTP opening in patients with acute myocardial infarction.

## Acknowledgments

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