Preconditioning of salvaged myocardium in conscious rabbits with postinfarction dysfunction

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ALTHOUGH IT IS WELL DOCUMENTED that early ischemic preconditioning strongly reduces myocardial infarct size induced by long periods of coronary artery occlusion (8), several studies have demonstrated that this effect is not associated with a concomitant improvement in regional contractile function during the first hours of reperfusion (6, 10). With longer periods of reperfusion, the recovery of myocardial function is enhanced in conscious preconditioned rabbits, but this recovery is disproportionately small compared with the degree of corresponding tissue salvage (3, 10). Because these reports investigated only one type of ischemic preconditioning, we hypothesized that different preconditioning protocols might induce differential functional recovery. Indeed, the critical effect of the number of occlusion-reperfusion cycle is widely recognized.

Accordingly, chronically instrumented conscious rabbits were subjected to a 30-min coronary artery occlusion (CAO) followed by 3 days of reperfusion (CAR) (13). Using this model, we compared the infantar-sparing and functional effects of two ischemic preconditioning stimuli, i.e., 1) one cycle of 5-min CAO and 10-min CAR and 2) six cycles of 4-min CAO and 4-min CAR. The functional measurements were performed using sonomicrometry. Finally, macrophage infiltration, apoptosis, and endothelial and inducible isoforms of nitric oxide synthase (eNOS and iNOS, respectively) expression were investigated using immunohistochemistry.

METHODS

The animal instrumentation and the ensuing experiments were performed in accordance with French official regulations.

Animal surgery. Male New Zealand White rabbits (2–2.5 kg) were anesthetized with a mixture of tiletamine (25 mg/kg iv) and zolazepam (25 mg/kg iv), intubated, and mechanically ventilated. Subsequent anesthesia was maintained with 2% isoflurane. An external electrocardiogram (ECG) was recorded during the surgery. A left thoracotomy was performed under sterile conditions at the fourth intercostal space. A pneumatic occluder manufactured from 18-gauge Tygon tubing was implanted around a major branch of the left coronary artery as previously described (13). A pair of 1-mm ultrasonic piezoelectric crystals (Sonometrics, London, ON, Canada) was inserted in the ventricular wall that would become ischemic with balloon inflation. The chest was closed in layers, and a small tube was left in the thorax to evacuate the air and fluids after surgery. Internal ECG leads were attached to the intercostal muscles. The occluder, internal ECG, and crystal wires were exteriorized between the scapulae. During the postoperative period, rabbits received buprenorphine (0.02 mg·kg⁻¹·day⁻¹·sc) and flunixin meglumine (1 mg·kg⁻¹·day⁻¹·im) for analgesia as well as gentamicin (0.5 mg·kg⁻¹·day⁻¹·im). Rabbits were allowed to recover for a minimum of 10 days after surgery.

Hemodynamic measurements. Data were digitized on a computer and analyzed using the data acquisition software HEM (v3.4; Notocord Systems, Croissy-sur-Seine, France). Heart rate was determined from the ECG recording. Regional segment length was measured by connecting the crystal wires to a sonomicrometer (TRX-4; Sonometrics). Percent segment shortening was calculated from the segment length recordings and defined as end-diastolic minus end-systolic segment lengths divided by end-diastolic segment length, multiplied by 100.

Experimental protocol. After recovery from surgery, rabbits were randomized into three groups: control, PC×6, and PC×1 (Fig. 1). The PC×6 group underwent a sequence of six successive 4-min CAO/4-

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adjacent microscopic fields encompassing the whole sample were For morphometry, all stained sections were observed with a micro-
sections were cut from each slice and stained with hematoxylin-eosin.
the crystals, we analyzed the formalin-fixed samples between the
area at risk or as a percentage of the left ventricle weight.
infarcted area was identified as
percentage of the left ventricle weight. Infarcted area was identified as
Determination of myocardial area at risk and infarct size. After
reperfusion, the animals received an injection of heparin and were anesthetized with pentobarbital sodium (50 mg/kg iv).
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min CAR cycles, and the PC×1 group was subjected to a single cycle
of 5-min CAO/10-min CAR. CAO and CAR were induced by man-
ually inflating and deflating the balloon occluder, respectively. After
completion of the preconditioning stimuli, the animals underwent a
30-min CAO followed by 72-h CAR. ECG and segment length were
recorded before (baseline) and throughout the preconditioning proto-
col as well as during the 30-min CAO and the first 3 h of subsequent
reperfusion. Additional recordings were performed at 24, 48, and 72 h of
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**Fig. 1. Experimental protocols.** PC×6, preconditioning with 6 cycles of 4-min coronary artery occlusion (CAO) and 4-min coronary artery reapertusion; PC×1, preconditioning with 1 cycle of 5-min CAO and 10-min coronary artery reapertusion.

**RESULTS**

Thirty chronically instrumented conscious rabbits were sub-
jected to CAO. Ten were excluded after ventricular fibrillation,
i.e., three control, four PC×6, and three PC×1 rabbits. Two
other rabbits, i.e., one control and one PC×1 rabbit, also were
excluded for technical reasons (lost of sonomicrometric con-
tractility signals). Finally, 18 rabbits successfully completed
the protocol: 6 control, 6 PC×6, and 6 PC×1 rabbits.

**Data analysis.** Values are expressed as means ± SE. Infarct sizes,
areas at risk, and ventricular weights were compared using one-way
ANOVA followed, if necessary, by a Student’s t-test with Bonferroni
correction. Heart rate and segment shortening values were compared
using two-way ANOVA with repeated measures followed, if neces-
sary, by Student’s t-test with Bonferroni correction. Significant
differences were determined as P < 0.05.

**Combined immunofluorescence for confocal laser microscopy.** To
assess the precise type of iNOS-expressing cells, we performed
double labeling of tissue sections as previously described (1, 2).
Briefly, anti-iNOS antibody was revealed in a first step by using
anti-rabbit biotinylated antibody and streptavidin-cyanin-2 (Amers-
ham, Les Ulis, France). In a second step, either anti-desmine (car-
diomyocyte marker) or anti-CD31 (endothelial cell marker) monoclo-
nal antibodies (Dako) were used and revealed using anti-mouse
antibody labeled with cyanin-3 (Amersham). The sections were ob-
served with a confocal microscope Leica TCS SP (Leica Microsys-
tems, Heidelberg, Germany). These experiments were performed in
sections from rabbits of the control and PC×1 groups, because no
iNOS expression was observed in the PC×6 group.

**Histochemical staining and morphometry.** To
investigate myocyte area at risk and ischemia,
infarct size, we analyzed the formalin-fixed samples between the
crystals using histology. As previously described (13), histological
sections were cut from each slice and stained with hematoxylin-eosin.
For morphometry, all stained sections were observed with a micro-
scope at a ×2 magnification. Successive digital photographs of the
adjacent microscopic fields encompassing the whole sample were
recorded. A computerized reconstruction (Photoline; Computerinsel,
Bad Göding, Germany) of the complete section at a ×2 magnification
was made by adequate juxtaposition of the different and comple-
mentary digital photographs. Infarct was delimited from this photo-
graphic reconstitution by drawing its contours with a computer mouse.
Simultaneous observation of the histological section on a microscope
at ×4 or ×10 magnification allowed an accurate detection of the
infarcted area, yielding high sensitivity in delimiting the infarcted
area. Myocardial infarction was considered a central region of coag-
ulation necrosis with a border of myocytolysis and inflammatory
infiltration in a granulation tissue. It was adjacent to myocardium
exhibiting a normal appearance and was considered living myocar-
dium. Finally, planimetry was performed, and infarct sizes between

crystals were measured.

**TdT-mediated dUTP nick end labeling and immunohistochemistry.**
Detection of apoptosis using the TdT-mediated dUTP nick end label-
ing (TUNEL) technique and of macrophages as well as eNOS and
iNOS expressions using immunohistochemistry was performed on
paraffin tissue sections from all rabbits that completed the experiment-
ental protocol. TUNEL ApopTag kit (Q. Biogene, Carlsbad, CA),
RAM11 antibody against rabbit macrophages diluted at 1:30 (Dako,
Trappes, France), and anti-iNOS and anti-iNOS antibodies diluted at
1:50 (Becton Dickinson Biosciences PharMingen, San Diego, CA) were
used in accordance with TUNEL or immunohistochemical procedures as previously described (1, 12).

**Graphical recording.** To determine myocardial infarction between
the crystals, we analyzed the formalin-fixed samples between the
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ward shift was significantly greater for the PC×6 compared with the PC×1 group.

Regional contractility. The baseline values of segment shortening were not significantly different among the three groups of rabbits (12.5 ± 3.0, 12.7 ± 0.7, and 14.3 ± 2.0% for control, PC×6, and PC×1 groups, respectively). As shown in Fig. 3, segment shortening was strongly and similarly depressed in the three groups throughout CAO and during the first 3 h of reperfusion, except at 5 min of CAR, when it was significantly greater in the PC×6 compared with the control group. From 24 to 72 h of CAR, segment shortening values were significantly enhanced in the PC×6 and PC×1 groups compared with the control group. At 48 and 72 h, the depression of segment shortening was significantly lower in the PC×6 (−42 ± 9 and −18 ± 10% from baseline, respectively) compared with the PC×1 group (−73 ± 4 and −68 ± 7% from baseline, respectively).

Histology. The overall histological pattern of the intercrystal myocardium sections was similar for all rabbits among the three groups. Indeed, all infarcts consisted of a core of patent myocyte coagulation necrosis with some residual hemorrhage and edema. They were limited by a clear-cut border of deter-
sion with granulation tissue and inflammation. Finally, no alteration was observed in the salvaged tissue. Individual infarct sizes between the crystals were plotted against the segment shortening recovery as shown in Fig. 4. A significant nonlinear inverse relationship was demonstrated between these two parameters ($y = \frac{-106}{e^{17/x}}; r = 0.87, P < 0.05$).

Infarct sizes also were specifically quantified in the subendocardium and in the subepicardium. Compared with those in control rabbits, subendocardial infarct sizes were significantly decreased in the PC×1 group (52 ± 8 vs. 28 ± 7%, respectively, $P < 0.05$) and, to a greater extent, in the PC×6 group (1 ± 1%, $P < 0.05$). However, subepicardial infarct sizes were similarly decreased in the PC×1 (4 ± 2%) and PC×6 groups (2 ± 1%) compared with the control group (24 ± 8%, $P < 0.05$).

TUNEL and immunohistochemistry. As shown in Fig. 5, the overall pattern regarding RAM11- and TUNEL-positive cells was similar in all myocardial intercrystal sections provided by the three groups. Macrophages defined as RAM11-positive cells and apoptotic cells defined as TUNEL-positive cells mainly were observed in the detersion border zone of infarction. In contrast, neither RAM11- nor TUNEL-positive cells were observed in the salvaged tissue.

Inducible NOS was differentially expressed among the three experimental groups. First, iNOS-positive cells were observed in the detersion border of infarction in macrophagic cells in the three groups. Second, intensive iNOS expression was observed...
in salvaged tissue in both the control and PC×1 groups but was almost absent in the PC×6 group. This expression was observed in the endothelium of large vessels and in the interstitium. This interstitial expression of iNOS appeared to be related to the microvascular endothelium, because no macrophage infiltration was observed in the salvaged tissue. In the three groups, no iNOS immunostaining was observed in the cytoplasm of salvaged cardiomyocytes. Finally, eNOS expression was similarly observed among groups in the endothelium of micro- and macrovessels. Combined immunofluorescence. No colocalization was observed between cardiomyocytes and iNOS expression (Fig. 6A), with iNOS expressed only in the interstitium adjacent to cardiomyocytes. Conversely, a colocalization was observed between iNOS expression and endothelial cells, in both large (Fig. 6B) and small coronary arteries (Fig. 6C) and in the microcirculation (Fig. 6D). This pattern was similarly observed in both the control and PC×1 groups. However, one cannot rule out iNOS synthesis in cardiomyocytes at very low levels, i.e., below the threshold of detection of immunohistochemistry.

DISCUSSION

The present study demonstrates that early ischemic preconditioning based on multiple brief ischemic episodes can induce an almost complete protection against postinfarction myocardial dysfunction in chronically instrumented conscious rabbits. Although it is widely recognized that early preconditioning strongly reduces infarct size after an ischemic insult, several studies have reported that this protective effect is not always associated with an improvement in postinfarction myocardial contractility (6, 10). These studies were limited, however, to the investigation of regional function within the first hours of reperfusion. In another study, Cohen et al. (3) reported in conscious rabbits that the functional protective effect of ischemic preconditioning could be revealed when reperfusion was extended to 72 h, i.e., segment shortening reached 44% of its baseline value with an infarct size averaging 10.2 ± 1.4%. Using the same preconditioning protocol (i.e., PC×1) in the present study, we confirmed this pattern, because segment shortening averaged 32 ± 7% of its baseline value with an infarct size reaching 15 ± 2%. Regarding these results, it could be hypothesized that preconditioning does not afford a strong and rapid protection against postinfarction dysfunction. One also could argue that the preconditioning protocol was not optimal. Indeed, by using six cycles of brief ischemia-reperfusion for preconditioning, our study demonstrates that an almost complete recovery in regional contractility can be achieved within 3 days of CAR. Under these conditions, segment shortening recovered up to 82 ± 9% of its respective baseline value and infarct size averaged 3 ± 1% of the area at risk. Although the difference in infarct sizes was rather modest between the two preconditioning protocols, the functional gain appeared dramatically potentiated with the use of multiple ischemia-reperfusion cycles. An additional 12% of viable tissue in PC×6 vs. PC×1 was indeed associated with an almost 50% gain in functional recovery at 72-h CAR compared with PC×1. One might argue that this could be explained by differences in the kinetics of functional recovery and prolonged stunning. This is unlikely, because with 21 days of CAR, Cohen et al. (4) reported a plateau in segment shortening recovery with PC×1 (64.7 ± 9.8% of baseline value), i.e., a value lower than that observed with PC×6 in the present study with 72-h CAR (82 ± 9% of baseline value). Therefore, compared with the preconditioning protocol with a single occlusion-reperfusion cycle, our results show that the use of six cycles allows us to reach both a more rapid and a greater contractile recovery of the postinfarcted zone despite modest differences in corresponding ultimate infarct sizes.

To further investigate whether this functional recovery was due to different infarct-sparing properties of the preconditioning sequences, we calculated an index corresponding to the ratio between segment shortening measured after 72-h CAR (percentage of baseline) and the amount of salvaged tissue (percentage of the intercysternal area). Such an index allows us to approximate the regional function of the sole salvaged tissue independently, theoretically, from the infarct size. For example, an index value of 1 indicates a complete recovery. In the control group, this index averaged 0.00 ± 0.10, indicating akinesis of salvaged cardiomyocytes. As expected, this index was greater with PC×1 (0.39 ± 0.07), demonstrating that, in agreement with previous results (4), preconditioning is able to induce a modest functional protection of the salvaged tissue along with its infarct-limiting effect. However, this functional protection can be further potentiated, because the index value was increased to 0.82 ± 0.09 by PC×6. Although the main determinant of postinfarction dysfunction remains infarct size, the use of such an index allows us to postulate that the two preconditioning protocols exert differential functional effects on salvaged tissue, i.e., independently of their infarct-sparing effects (9, 11).

Several hypotheses might be raised to explain this apparent differential effect of these preconditioning protocols. Tissue loss due to infarction is known to have differential consequences when localized in the subendocardium vs. subepicardium. Indeed, infarct size in the subendocardium was dramatically reduced in the PC×6 vs. the PC×1 group, a result that...
Fig. 6. Combined immunofluorescence. iNOS expression (left) was matched to cell markers to determine precisely which cell type actually produced iNOS. Detection of iNOS is indicated in green (cyanin-2). Detection of desmine and CD31 is indicated in red (cyanin-3). No colocalization was observed with the cardiomyocyte marker desmine (A). A colocalization was observed with the endothelial cell marker CD31 in large-sized coronary arteries (B), in small-sized coronary arteries (C), and in microcirculation (D). Bars = 50 μm in A and B; bars = 25 μm in C and D. Images were taken from a control rabbit heart. The observed pattern was similar in PC1 hearts.
confirms that localization and geometry of infarction may induce a nonlinear relationship between infarct size and postinfarction dysfunction. Another consequence of infarction on the peri-infarct viable tissue is a paradoxical lengthening in the early systole, i.e., mechanical tethering (7). In the present study, a potent tethering was indeed observed in both the control and PC×1 groups but not in the PC×6 group (1.8 ± 0.8, 1.4 ± 0.6, and 0 ± 0% of the telediastolic length, respectively). However, other factors not directly dependent on infarct size are also probably involved in postinfarction regional dysfunction. For example, our immunohistochemistry and colabeling immunofluorescence experiments demonstrated that iNOS is highly expressed in the endothelium of the coronary vessels of both the control and PC×1 groups but not in the PC×6 group. This clearly shows that biochemical changes occur within the salvaged tissue that may partly explain the difference between preconditioning protocols. Indeed, it is known that activation of iNOS during reperfusion may contribute to myocardial dysfunction through a negative inotropic effect (5, 14) and that inhibition of iNOS improves the functional recovery of postinfarcted myocardium in rabbits (14). One can therefore speculate that PC×6 allows, at least in part, a greater functional recovery of salvaged tissue by preventing iNOS expression and upregulation during the postinfarction period. It also should be acknowledged that differences in the kinetic of iNOS expression during reperfusion could also explain part of our results. Finally, we can rule out changes in apoptosis, macrophage infiltration, and eNOS expression in the salvaged tissue because they were not observed in all rabbits in the salvaged tissue.

In conclusion, this study demonstrates for the first time to our knowledge that early ischemic preconditioning can confer a rapid and almost complete protection against postinfarction dysfunction when it is induced by multiple vs. unique ischemia-reperfusion cycles. This protection not only involves an increase of the amount of viable tissue but also exerts a beneficial effect on the functional recovery of the salvaged myocardium. Inhibition of iNOS might represent a potential target for protecting the salvaged reperfused myocardium against postinfarction dysfunction. Further specific experiments with iNOS inhibitors are required to strengthen this conclusion. This work, however, was beyond the scope of the present study, which was not designed to perform such investigations.

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