Preconditioning delays Ca\(^{2+}\)-induced mitochondrial permeability transition

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Abstract

Objective: We investigated whether ischemic preconditioning (PC) may modify mitochondrial permeability transition (MPT) pore opening. Methods: In protocol 1, New Zealand White rabbits underwent either no intervention (sham group) or 10 min of ischemia followed by 5 min of reperfusion, preceded (PC) or not (C; control) by one episode of 5 min of ischemia and 5 min of reperfusion. Rabbits were pretreated by either saline or the MPT pore inhibitor cyclosporin A (CsA), or its non-immunosuppressive derivative Cs29 (10 mg/kg, IV bolus). Hearts were harvested and mitochondria isolated for further assessment of Ca\(^{2+}\)-induced MPT using a Ca\(^{2+}\)-sensitive micro-electrode. In protocol 2, C and PC hearts underwent 30 min of ischemia and 4 h of reperfusion. They were pretreated either by saline, CsA or Cs29, as in protocol 1. Infarct size was assessed by triphenyltetrazolium, and apoptosis by TUNEL staining. Results: In protocol 1, the Ca\(^{2+}\) overload required to induce MPT pore opening was significantly higher in PC than in C hearts. CsA and Cs29 significantly increased the Ca\(^{2+}\) overload required for MPT pore opening. In protocol 2, mean infarct size averaged 25% of the risk region in CsA/Cs29 treated hearts versus 15% in PC and 55% in controls (\(P<0.05\) vs. C, \(P=ns\) vs. PC). Cardiomyocyte apoptosis was significantly reduced by PC and cyclosporin treatment with a mean apoptotic index of less than 2% in either group versus more than 11% in controls. Conclusion: This suggests that delayed opening of MPT pore may play a major role in ischemic PC.

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Keywords: Preconditioning; Calcium (cellular); Mitochondria; Apoptosis; Necrosis

1. Introduction

Ischemic preconditioning (PC) has been shown to limit both necrotic and apoptotic cardiomyocyte cell death [1–4]. Yet, the mechanism of this cardioprotection remains unclear. Ischemia-reperfusion affects various key mitochondrial functions, including ATP production, Ca\(^{2+}\) homeostasis, oxygen-derived free radical production, and permeability transition [5]. Mitochondrial permeability transition (MPT) is due to opening of a large pore in the inner mitochondrial membrane, whose structure remains incompletely known [6]. MPT pore opening causes matrix swelling, inner membrane potential (\(\Delta \Psi_m\)) collapse, uncoupling of the respiratory chain, efflux of Ca\(^{2+}\) and release of small proteins such as cytochrome c [7]. MPT is mostly induced by Ca\(^{2+}\) overload, especially when it is associated with adenine nucleotide depletion, increased inorganic phosphate concentration, oxidative stress, all features of ischemia-reperfusion [8]. MPT pore opening is inhibited by the immunosuppressive drug cyclosporin A (CsA) that can protect the heart from ischemia-reperfusion in in vitro models [9–13]. MPT appears to be a critical event in the transition from reversible to irreversible myocardial injury following an ischemic insult [14].

Whether ischemic PC alters MPT pore opening, and whether this may explain its antinecrotic and antiapoptotic effects remains unclear. Therefore, our objective was to determine: (1) whether mitochondria, directly isolated from preconditioned hearts, display any alteration of Ca\(^{2+}\)-induced MPT pore opening, and (2) whether in vivo pharma-
collogial reproduction of this in vitro alteration might protect the ischemic-reperfused heart from necrosis and apoptosis.

2. Methods

2.1. Surgical preparation


Male New Zealand White rabbits, weighing 2.2–2.5 kg were anesthetized by intramuscular injections of xylazine (5 mg/kg) and ketamine (50 mg/kg), as previously described [15]. An intravenous infusion of a mixture of xylazine (20–50 μg/kg per min) and ketamine (40–100 μg/kg per min) was then maintained throughout the experiment. After a midline cervical incision, a tracheotomy was performed and animals were ventilated with room air. 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. After an intravenous bolus administration of fentanyl (10 mg/kg), a left thoracotomy was performed in the fourth left intercostal space. Despite the fact that opioids can protect the ischemic heart, administration of fentanyl, likely did not alter the results since it was performed in a similar way in all experimental groups. The pericardium was opened and the heart exposed. A 3.0 size silk suture attached to a small curved needle was passed around a marginal branch of the left circumflex coronary artery. Both ends of the thread were passed through a small vinyl tube to form a snare that could be tightened to occlude and loosened to reperfuse the artery. Body temperature was maintained throughout the experiment. After a midline cervical incision, a tracheotomy was performed and animals were ventilated with room air. 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. After an intravenous bolus administration of fentanyl (10 mg/kg), a left thoracotomy was performed in the fourth left intercostal space. Despite the fact that opioids can protect the ischemic heart, administration of fentanyl, likely did not alter the results since it was performed in a similar way in all experimental groups. The pericardium was opened and the heart exposed. A 3.0 size silk suture attached to a small curved needle was passed around a marginal branch of the left circumflex coronary artery. Both ends of the thread were passed through a small vinyl tube to form a snare that could be tightened to occlude and loosened to reperfuse the artery. Body temperature was monitored via an intraperitoneal thermometer and kept constant by means of a heating pad. Heart rate and arterial pressure were monitored continuously throughout the experiment on a Gould® recorder (Gould Inc., Cleveland, OH). After the surgical procedure, a 15-min stabilization period was observed.

2.2. Protocol 1: Ca\textsuperscript{2+}-induced MPT pore opening in preconditioned heart

Protocol 1 investigated whether mitochondria isolated from preconditioned hearts display a different pattern of Ca\textsuperscript{2+}-induced MPT pore opening than controls and, if so, how it compares to that of cyclosporin-treated hearts.

2.2.1. Experimental design

Seventy-six rabbits were included in this protocol and randomly assigned to one of nine groups (Fig. 1). Sham animals underwent no ischemia for 30 min \( (n=26) \). All control or preconditioned rabbits underwent a 10-min coronary artery occlusion followed by 5 min of reperfusion. The 10-min duration was chosen according to pilot experiments indicating that, on one hand, it was short enough not to induce any irreversible myocardial injury, and on the other hand, because it was long enough to alter MPT pore opening in this preparation. Prior to this, control rabbits underwent no intervention for 15 min (control groups, C: \( n=25 \)), while preconditioned received 5 min of ischemia followed by 5 min of reperfusion (preconditioned groups, PC: \( n=25 \)). At the onset of the 30-min experiment, all rabbits received an intravenous bolus of either saline (sham: \( n=10, C: n=9, PC: n=9 \)), cyclosporin A (CsA, 10 mg/kg) (Sham-CsA, C-CsA, PC-CsA: \( n=8 \) per group) or cyclosporin 29 (Cs29, 10 mg/kg) (Sham-Cs29, C-Cs29, PC-Cs29; \( n=8 \) per group). At the end of the experiment, hearts were excised while still beating, and mitochondria isolated from the myocardium at risk for further assessment of Ca\textsuperscript{2+}-induced MPT pore opening.

2.2.2. Preparation of isolated mitochondria

Preparation of mitochondria was adapted from a previously described procedure [16,17]. All operations were carried out in the cold. Heart pieces (0.5–1.0 g) were placed in isolation buffer A containing 70 mM sucrose, 210 mM mannitol, 1 mM EDTA in 50 mM Tris/HCl pH 7.4. The tissue was finely minced with scissors and then homogenized in the same buffer (10 ml buffer per g tissue), using
successively a Kontes tissue grinder and a Potter Elvejem. The homogenate was centrifuged at 1300 × g for 3 min. The supernatant was poured through cheesecloth and centrifuged at 10,000 × g for 10 min. The mitochondrial pellet was resuspended in isolation buffer B containing 70 mM sucrose, 210 mM mannitol, 0.1 mM EDTA in 50 mM Tris/HCl pH 7.4. After aliquots were removed for protein measurements, the mitochondria (by aliquots of 6 mg proteins) were washed in isolation buffer B, centrifuged at 6800 × g for 10 min and stored as pellets on ice prior to MPT pore opening experiments. Protein content was routinely assayed according to Gornall’s procedure using bovine serum albumin as a standard [18]. Purity and integrity of isolated mitochondria were assessed by measuring specific activities of monoamine oxidase (EC 1.4.3.4), as an outer membrane marker enzyme, and cytochrome c oxidase (EC 1.9.3.1), as an inner membrane marker enzyme [19,20].

2.2.3. Ca2+-induced MPT pore opening

MPT pore opening was assessed following in vitro Ca2+ overload. Isolated mitochondria (6 mg proteins) were suspended in 100 μl buffer B, and added in 900 μl of buffer C (150 mM sucrose, 50 mM KCl, 2 mM KH2PO4, 5 mM succinic acid to 20 mM Tris/HCl pH 7.4) within a Teflon chamber equipped with a Ca2+-selective microelectrode, in conjunction with reference electrode [21,22]. Modifications of the medium (i.e. extra-mitochondrial) Ca2+ concentration were continuously recorded using a custom made Synchroline® software. Mitochondria were gently stirred for 1.5 min. At the end of the pre-incubation period, 20 μM CaCl2 administration was performed every 60 s. As depicted in Fig. 2, each 20 μM CaCl2 administration was performed as a peak of extramitochondrial Ca2+ concentration. Ca2+ is then very rapidly taken up by the mitochondria resulting in a return of extramitochondrial Ca2+ concentration to near baseline level. Following sufficient Ca2+ loading, extramitochondrial Ca2+ concentration abruptly increases indicating a massive release ofCa2+ by mitochondria due to MPT pore opening (Fig. 2). The amount of Ca2+ necessary to trigger this massive Ca2+ release is used here as an indicator of the susceptibility of MPT pore to Ca2+ overload.

2.2.4. Electron microscopy

Electron microscopy was performed either at the end of the pre-incubation period (i.e. before Ca2+ loading), or at the end of the experiment, following either no intervention (to address alteration of the preparation), or Ca2+ overload (i.e. after MPT pore opening). Under each of these three experimental conditions, samples of mitochondria were fixed for 2 h in 2% glutaraldehyde, 100 M phosphate buffer, pH 7.4 and postfixed in 1% osmium tetroxide. Dehydration was performed in a series of ethanol and propylene oxide extractions, before sample embedding in Epon.

2.3. Protocol 2: cardioprotective effects of MPT pore opening inhibition in vivo

Based on the in vitro results of protocol 1, regarding the respective MPT pore opening profiles of ischemic PC and cyclosporin, we performed protocol 2 to compare the anti-necrotic and antiapoptotic effects of both types of intervention in the in situ rabbit heart.

2.3.1. Experimental design

Fifty-nine rabbits were included in this protocol and randomly assigned to one of six groups (Fig. 1). All animals underwent a 30 min prolonged coronary artery occlusion followed by 4 h of reperfusion. Prior to this, rabbits underwent either no intervention (control groups; n = 31), or PC by 5 min ischemia followed by 5 min of reperfusion (preconditioned groups; n = 28). All rabbits received an intravenous bolus of either saline (C: n = 12, and PC: n = 12), cyclosporin A (CsA, 10 mg/kg) (C-CsA: n = 12 and PC-CsA: n = 9) or cyclosporin 29 (Cs29, 10 mg/kg) (C-Cs29: n = 7 and PC-Cs29: n = 7), 15 min before the prolonged ischemia. In these six groups, hearts were harvested at the end of the 4-h reperfusion period for further assessment of infarct size and myocardial apoptosis (this latter technique was performed in seven animals per group).

2.3.2. Area at risk and infarct size determination

At the end of the 4-h reperfusion, the coronary artery was briefly reoccluded and 0.5 mg/kg Uniperse blue pigment (Ciba–Geigy®, Hawthorne, NY) was injected intravenously to delineate the in vivo area at risk (AR), as previously described [23]. With this technique, the previously non-ischemic myocardium appears blue, whereas the previously ischemic myocardium (AR) remains unstained. Anesthetized rabbits were then euthanized by an intravenous injection of 4 mEq KCl. The heart was
excised and cut into five to six 2 mm thick transverse slices, parallel to the atroventricular groove. After removing right ventricular tissue, each heart slices was weighed. The basal surface of each slice was photographed for later measurement of the AR. Each slice was then incubated for 15 min in a 1% solution of triphenyltetrazolium chloride at 37 °C to differentiate infarcted (pale) from viable (brick red) myocardial area [24]. The slices were then rephotographed. Enlarged projections of these slices were traced for determination of the boundaries of the AR and area of necrosis (AN). Extent of the AR and AN was quantified by computerized planimetry and corrected for the weight of the tissue slices. Total weights of the AR and the AN were then calculated and expressed in grams and as percentage of total left ventricle (LV), and of the AR weight, respectively. We decided prospectively that hearts with a risk region less than 10% of the LV weight would be excluded from the study.

2.3.3. Myocardial apoptosis

The TUNEL assay uses a terminal deoxynucleotidyl transferase (TdT) to label free 3′ OH ends in genomic DNA, and thus localizes and assesses cells undergoing DNA fragmentation [25]. Freshly frozen non-ischemic and ischemic myocardial samples were cut (5 μm thickness), mounted on silanized glass slides, fixed with 4% paraformaldehyde for 1 h at room temperature. The sections were washed in PBS. Endogenous peroxide activity was quenched by a 30-min incubation in 3% hydrogen peroxide in methanol at room temperature. The heart slices were washed in PBS, and permeabilized for 2 min at 4 °C in 0.1% triton X-100 in 0.1% sodium citrate (Sigma®). Proteins were removed from the tissue sections by incubation for 30 min at 37 °C in 20 μg/ml proteinase K (Sigma®). DNA fragments in the tissue sections were determined using an in situ cell death detection kit (Boehringer Mannheim®, Switzerland). After washing in PBS, sections were incubated for 60 min at 37 °C in a humid chamber with the TUNEL reaction mixture supplied by the kit and containing TdT and fluoresein-dUTP. During this incubation step, TdT catalyzes the attachment of fluorescein-dUTP to the free 3′ OH ends in the DNA. After washing with PBS, coverslips were mounted. Slides incubated without TdT were used as negative controls. Slides were examined with a Leica® fluorescence microscope. For each slide, 10 separate fields were analyzed by two independent observers using a × 40 objective. Cardiomyocytes with green nuclear fluorescence were defined as TUNEL positive cells. The mean percentage of TUNEL positive cardiomyocytes was calculated and expressed as the number of TUNEL positive cells relative to the total number of myocytes (nuclei).

2.4. Chemicals

Both forms of cyclosporin used in the present study were a generous gift of Novartis® (Basel, Switzerland). CsA and its non-immunosuppressive derivative [Me-Ile4]-cyclosporin (cyclosporin 29, Cs29) were used either in vivo (10 mg/kg, IV) or in vitro (0.25, 0.50, 1, 2, and 5 μM). CsA and Cs29 were dissolved in ethanol and diluted in buffer C prior to utilization, in order to obtain for each dilution the equivalent of 2.5 μl pure ethanol in 1 ml for in vitro experiment. The effect of this amount of pure ethanol was evaluated in the same conditions. For in vivo use, CsA and Cs29 were dissolved in a mixture of Cremophor EL (polyethoxylated castor oil) with ethanol-94%.

2.5. Statistical analysis

Comparisons between groups were performed using one-way analysis of variance (ANOVA) and, when a significant F value was obtained, means were compared using a Tuckey’s test. Differences in the relationship between infarct size and area at risk were evaluated by analysis of covariance (ANCOVA) and post hoc Tuckey’s test, with infarct size as the dependent variable and area at risk as the covariant. All values are expressed as mean ± standard error (S.E.M.). Statistical significance was defined as a value of P<0.05.

3. Results

One hundred forty-four rabbits were included in the present study: 80 in protocol 1 and 64 in protocol 2. Nine rabbits were excluded: four in protocol 1, because of technical failure during evaluation of MPT pore opening, and five in protocol 2 (three because of cardiacogenic shock during sustained ischemia or unexplained death during reperfusion and two because of a smaller risk region). Results are then presented for the remaining 135 rabbits.

3.1. Protocol 1: Ca2+-induced MPT pore opening in preconditioned heart

3.1.1. Isolated mitochondria preparation

In all cases, mitochondrial suspensions exhibited high monoamine oxidase and cytochrome c oxidase specific actives (i.e. specific mitochondrial marker enzymes) (data not shown). The quality of the preparation was further demonstrated by electron microscopy. Fig. 3A depicts isolated mitochondria suspended in buffer C, i.e. just before Ca2+- loading. Clearly, mitochondria display intact membranes and dense matrix space. Comparable morphology was observed in the sham group at the end of the protocol, indicating that the preparation did not alter over the time of the experiment (data not shown). In contrast, following Ca2+-induced MPT pore opening, most mitochondria underwent dramatic morphological changes, as we observed large swollen mitochondria,
with disruption of outer membrane and disappearance of the cristae (Fig. 3B).

We evaluated in vitro the effects of increased concentrations of either CsA or its non-immunosuppressive derivative Cs29 on Ca\(^{2+}\)-induced MPT pore opening in mitochondria isolated from sham hearts. Exposure to CsA during 1 min before the first 20 \(\mu\)M Ca\(^{2+}\) pulse, using concentrations ranging from 0.25 to 1 \(\mu\)M, significantly and dose dependently delayed Ca\(^{2+}\)-induced MPT pore opening when compared to untreated sham hearts (data not shown). This demonstrated that the abrupt Ca\(^{2+}\) release was indeed due to MPT pore opening. The presence of 2.5 \(\mu\)l ethanol, i.e. the amount required to dissolve CsA, did not modify mitochondrial Ca\(^{2+}\) uptake and release.

### 3.1.2. Effect of ischemia-reperfusion on Ca\(^{2+}\)-induced MPT pore opening

In the sham group, the amount of Ca\(^{2+}\) required to open the MPT pore averaged 308 ± 20 \(\mu\)M. This Ca\(^{2+}\) overload was significantly reduced in the control group, averaging 165 ± 22 \(\mu\)M (\(P<0.05\) vs. sham). In preconditioned hearts, the Ca\(^{2+}\) overload required for MPT pore opening significantly increased when compared to controls, averaging 300 ± 34 \(\mu\)M (\(P<0.05\) vs. control, \(P=ns\) vs. sham) (Fig. 4).

In all groups, in vivo pretreatment by CsA or Cs29 resulted in a significant increase in the Ca\(^{2+}\) overload required to induce MPT pore opening (Fig. 4). There was no difference among CsA and Cs29-treated groups.

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**Fig. 3.** Mitochondria morphology following Ca\(^{2+}\)-induced MPT pore opening. Electron microscopy confirmed the integrity and purity of mitochondrial fraction before Ca\(^{2+}\)-induced MPT pore opening (A). Following Ca\(^{2+}\)-induced MPT pore opening, mitochondria appeared swollen with disappearance of membrane integrity (B).

**Fig. 4.** Ca\(^{2+}\) overload required for MPT pore opening in preconditioned and cyclosporin-treated hearts. Ca\(^{2+}\) overload required for MPT pore opening in protocol 1 (as percent of sham values). In the control group, Ca\(^{2+}\) overload required for MPT pore opening was significantly reduced vs. sham animals. PC increased Ca\(^{2+}\) load to near sham values. Mitochondria isolated from animals treated with either CsA or Cs29 were particularly resistant to Ca\(^{2+}\) overload. Full bars, no pretreatment; empty bars, CsA-pretreated; striped bars, Cs29 pretreated rabbits. C, control; PC, preconditioned. \(*P<0.05\) vs. sham. \(1P<0.05\) vs. C. \(2P<0.05\) vs. PC.

**Fig. 5.** Effect of cyclosporin and PC on infarct size. AN is expressed in percent of AR. As expected, PC significantly reduced infarct size vs. the control group (C). Similarly, all cyclosporin-treated control (C-CsA and C-Cs29) or preconditioned (PC-CsA and PC-Cs29) rabbits developed smaller infarct than controls. \(*P<0.05\) vs. C.
exposed to a Ca\(^{2+}\) overload. Moreover, ischemic PC and the MPT pore inhibitor cyclosporin, that causes a PC-like in vitro pattern of Ca\(^{2+}\)-induced MPT pore opening, provide a strong antinecrotic and antiapoptotic protection when administered in vivo.

4.1. Ca\(^{2+}\)-induced MPT pore opening in preconditioned hearts

We used a quantitative potentiometric approach to address the susceptibility of the MPT pore to open following Ca\(^{2+}\) loading, in purified mitochondria that were directly isolated from in vivo injured myocardium [22,26,27]. In vitro use of Cs29, that is devoid of the calcineurin-dependent immunosuppressive action of CsA, but conserves its effects on mitochondrial cyclophilin D (i.e. highly specific for the MPT pore), confirmed that the abrupt in vitro Ca\(^{2+}\) release from isolated mitochondria actually reflects MPT pore opening [28].

Using an isolated rat heart preparation, Hausenloy et al. and Javadov et al. recently suggested that suppression of MPT pore opening may be important for PC [5,29]. Others reported that, in the settings of ischemia-reperfusion, MPT pore activity can be modulated by mediators of PC like mitochondrial cyclophilin D (i.e. highly specific for the MPT pore), confirmed that the abrupt in vitro Ca\(^{2+}\) release from isolated mitochondria actually reflects MPT pore opening [28].

We demonstrated that a single episode of reversible ischemia (i.e. 10 min) significantly alters Ca\(^{2+}\)-induced MPT pore opening. Ca\(^{2+}\)-induced MPT pore opening was assessed following such a reversible ischemic insult, in order to avoid isolating mitochondria from heterogeneously infarcted tissue with a mixture of dead and still viable cardiomyocytes. After 30 min of ischemia and 4 h of reperfusion, surviving cardiomyocytes (i.e. mitochondria) come from the least ischemic part of the AR in control hearts, but from a larger and more severely ischemic risk region in myocardium salvaged by PC: those two populations of mitochondria are, therefore, not truly comparable. This problem can only be avoided by excising myocardium before any irreversible injury, as was performed in protocol 1 of the present study.

Mitochondria directly isolated from preconditioned hearts required a significantly higher Ca\(^{2+}\) loading than controls to open the MPT pore. Ca\(^{2+}\)-induced MPT pore opening in mitochondria isolated from preconditioned hearts resembled that of mitochondria isolated from cyclosporin-treated control hearts, with in both cases a significant delay of pore opening. This was still true when Cs29, devoid of any action on calcineurin but specific for the MPT pore, was used. The larger delay observed in cyclosporin-treated

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3.2. Protocol 2: infarct size and apoptosis

3.2.1. Infarct size

Heart rate and blood pressure were not significantly different among the six groups of animals (data not shown). AR was comparable among the six groups of rabbits, with mean values averaging 24 ± 3%, 27 ± 2%, 32 ± 2%, 29 ± 3%, 32 ± 3%, and 37 ± 2% of the LV weight, in C, C-CsA, C-Cs29, PC, PC-CsA and PC-Cs29, respectively (P= ns among the six groups). PC significantly reduced infarct size that averaged 15 ± 4% of the AR versus 55 ± 8% in the control group (P< 0.05). Similarly, all cyclosporin-treated (CsA or Cs29) rabbits developed significantly smaller infarcts than controls. AN of C-CsA, C-Cs29, PC-CsA and PC-Cs29 averaged 24 ± 4%, 26 ± 6%, 24 ± 6%, 25 ± 5% of the risk region, respectively (P<0.01 vs. control group; P= ns vs. PC group) (Fig. 5). ANCOVA confirmed this significant difference among ischemic preconditioned or cyclosporin-treated hearts on one hand, and control hearts on the other hand. There was no difference among preconditioned groups and cyclosporin-treated groups.

3.2.2. Myocardial apoptosis

The percentage of TUNEL positive cardiomyocytes in the AR was significantly reduced in the PC group (1.9 ± 0.9%) when compared to the control group (11.8 ± 4.2%) (Fig. 6). Similarly, it averaged 1.8 ± 0.7%, 0.8 ± 0.6%, 0.6 ± 0.5% and 0.8 ± 0.4% in C-CsA, PC-CsA, C-Cs29 and PC-Cs29 groups, respectively (P<0.05 vs. control).

4. Discussion

In the present study, we report for the first time that mitochondria directly isolated from in vivo preconditioned rabbit hearts display a delayed MPT pore opening when

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Fig. 6. Cardiomyocyte apoptosis. TUNEL positive cardiomyocytes in the risk region. Full bars, no pretreatment; empty bars, CsA-pretreated; striped bars, Cs29 pretreated rabbits. C, control; PC, preconditioned. *P<0.01 vs. C.
versus preconditioned hearts is likely related to a dose effect, since cyclosporin blocks the MPT pore in a dose-dependent manner. It is possible that a smaller dose of CsA would still limit cell death and block Ca\(^{2+}\)-induced MPT pore opening for a value of Ca\(^{2+}\) load much closer to that of the preconditioned group; such an approach might give a better estimate of the involvement of MPT pore opening in the protective effect of ischemic PC and cyclosporin pre-treatment. In addition, the specific role of several factors that influence MPT pore opening, including membrane potential, pH and reactive oxygen species likely play a role in the present study and deserve further investigations. These observations strongly suggest that ischemic PC protect the mitochondria by influencing MPT pore opening.

How ischemic PC might alter MPT pore opening remains to be determined, although it has been proposed that activation of mitochondrial K\(_{\text{ATP}}\) channels during ischemic PC would drop membrane potential, reduce mitochondrial Ca\(^{2+}\) uptake and thereby limit induction of MPT pore opening [22,34]. One might hypothesize that matrix Ca\(^{2+}\) concentration might be reduced in mitochondria isolated from preconditioned hearts before in vitro Ca\(^{2+}\) loading. Using Indo-1 fluorescence in isolated rat hearts, Wang et al. reported that ischemic PC decreases mitochondrial Ca\(^{2+}\) concentration following 25 min of global ischemia and 30 min of reperfusion [35]. Whether this applies to the present study is, however, uncertain, since following 10 min of ischemia plus reperfusion, cell (and mitochondrial) Ca\(^{2+}\) overload is usually very limited [36]. Inorganic phosphate, protons, K\(^{+}\), ATP, radical oxygen species are modulators of the MPT pore, especially under ischemia-reperfusion conditions. There is evidence that PC may preserve mitochondrial oxygen consumption capacity and ATP production, and limit the production of oxygen-derived free radicals at reperfusion [37–39]. Whether modifications of one or several of these factors in preconditioned mitochondria might explain the delayed Ca\(^{2+}\)-induced MPT pore opening observed in the present study, requires further investigations.

4.2. Delaying Ca\(^{2+}\)-induced MPT pore opening protects the in vivo ischemic heart

Because the pattern of Ca\(^{2+}\)-induced MPT pore opening in mitochondria isolated from cyclosporin-treated hearts resembled that of preconditioned mitochondria, we decided to investigate whether CsA would mimic in vivo ischemic PC, i.e. reduce both infarct size and apoptosis. The anti-necrotic effect of cyclosporin has previously been reported, mainly in isolated rat heart preparations [9–12]. In the present study, we extended this observation to the in vivo rabbit model of myocardial infarction. In addition, we report that (1) reduction in infarct size was comparable in preconditioned and cyclosporin-treated rabbits, (2) PC and cyclosporin did not appear to have an additive beneficial effect on infarct size reduction, and (3) Cs29 and CsA afforded comparable antinecrotic effect. The antinecrotic effect of Cs29 clearly indicates that MPT pore opening is a key event in necrotic cardiomyocyte death. The fact that cyclosporin and ischemic PC did not have any additive effect on infarct size may indirectly suggest that they both act on MPT pore opening.

MPT pore opening is known to cause cytochrome c release which activate downstream caspases to further execute DNA fragmentation, i.e. apoptotic cell death [11]. In agreement with previous studies, we report that PC dramatically reduces cardiomyocyte apoptosis [2–4]. Cs29 and CsA also attenuated apoptosis, confirming that MPT pore opening is a key event of the death process consecutive to prolonged myocardial ischemia-reperfusion injury [8,40]. Ischemic PC has been shown to limit production of oxygen-derived free radicals, increase expression of the antiapoptotic protein Bcl-2, decrease expression of the proapoptotic protein Bax, or limit production of the secondary messenger ceramide, all factors known to directly affect MPT pore opening [4,39,41,42]. Whether modification of one or several of these factors by ischemic PC may explain the modulation of MPT pore opening and the consecutive attenuation of apoptosis observed in the present study requires further investigation. In any case, these in vivo data closely parallel isolated mitochondria results, and demonstrate that ischemic PC alters Ca\(^{2+}\)-induced MPT pore opening and subsequent cardiomyocyte apoptosis.

The results of the present study indicate that PC affects Ca\(^{2+}\)-induced MPT pore opening, and that inhibiting pore opening in vivo strongly protects the ischemic heart against both necrotic and apoptotic cell death.

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