Adenosine A₁-receptor induced late preconditioning and myocardial infarction: reperfusion duration is critical

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We investigated the influence of coronary artery reperfusion (CAR) duration on the infarct-limiting properties of adenosine A₁-receptor stimulation-induced delayed preconditioning (A₁-DPC) compared with ischemia-induced delayed preconditioning (I-DPC). Sixty-one chronically instrumented conscious rabbits successfully underwent the following protocol. On day 1, rabbits were randomly divided into four groups: control (saline, iv), I-DPC (six 4-min coronary artery occlusion/4-min reperfusion cycles), A₁-DPC (six 2-mercaptopropionyl glycine (14). Therefore, it may be hypothetized that the duration of posts ischemic coronary artery reperfusion could also be critical for the infarct-limiting effect of A₁-DPC.

Accordingly, the aim of this study was to compare the cardioprotective effects of A₁-DPC after short (3 h) and long (72 h) durations of reperfusion. We also performed a similar investigation with I-DPC. To avoid the confounding effects of numerous factors associated with the open-chest state, such as anesthesia, hypothermia, trauma, and elevated catecholamines, we performed this study in a model of conscious chronically instrumented rabbits.

METHODS

The animal instrumentation and the ensuing experiments were performed in accordance with the official regulations edicted by the French Ministry of Agriculture (Agreement no. A94-043-12).

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) and intubated and mechanically ventilated with 100% oxygen via a positive pressure respirator. The ventilation rate was 25 breaths/min, and the tidal volume was ~25 ml. Subsequent anesthesia was maintained with pentobarbital sodium (20–30 mg/kg iv). An external electrocardiogram (ECG) was recorded during the surgery. A left thoracotomy was performed at the fourth intercostal space under sterile conditions. A pulmonary occluder fashioned from 18-gauge Tygon tubing was implanted...
around a major branch of the left coronary artery according to a technique previously described (6). Proper functioning of the occluder was confirmed by observing cyanosis of the distal myocardium and ST segment deviation of the ECG after a brief inflation of the occluder. Conversely, hyperemia and normalization of the ECG were verified after its deflation. The chest was closed in layers, and a small tube was left in the thorax to evacuate air and fluids after surgery. Internal ECG leads were attached to intercostal muscles. The occluder and internal ECG wires were exteriorized between the scapulae. During the postoperative period, rabbits were treated for 3 days with buprenorphine (0.02 mg/kg sc) and flunixin meglumine (1 mg/kg im) for analgesia. Gentamycin (0.5 mg/kg im) was also administered during 5 consecutive days. Rabbits were allowed to recover for a minimum of 10 days after surgery.

Experimental protocol. Throughout the experiment, rabbits were conscious and kept in a box in a quiet, dimly lit room. The internal ECG wires were connected to an amplifier (Gould Instruments; Cleveland, OH). An intra-arterial catheter was introduced into the ear artery, and arterial pressure was measured using a Statham P23 ID strain gauge transducer (Statham Instruments; Oxnard, CA). ECG and arterial pressure were recorded on a multichannel oscillograph (DMS 1000, Graphitec, Vanderbilt; Irvine, CA).

Rabbits were randomly assigned to one of four groups: control, I-DPC, A1-DPC100, and A1-DPC400. The protocol was realized during 2 consecutive days, i.e., 24 h apart as illustrated in Fig. 1. On day 1, the control, A1-DPC100, and A1-DPC400 groups received an intravenous (ear vein) bolus injection (5 ml) of saline, 100 and 400 µg/kg N6-cyclopentyladenosine (CPA, Sigma Aldrich; Steiheim, Germany), respectively. As previously reported (26), these two doses of CPA were chosen on the basis of preliminary studies as being the ED50 and ED80, respectively. As previously reported (26), these two doses of CPA were chosen on the basis of preliminary studies as being the ED50 and ED80, respectively, at decreasing mean arterial pressure. The I-DPC group underwent a sequence of six preconditioning, 400 µg/kg CPA, which lasted either 3 or 72 h. If ventricular fibrillation occurred, no defibrillation was attempted, and the rabbits were rapidly euthanized.

Determination of myocardial area at risk and infarct size. After reperfusion was completed, animals received an injection of heparin (1,000 IU iv) and were reanesthetized with pentobarbital sodium (50 mg/kg iv). Potassium chloride was administered intravenously to induce cardiac arrest. The hearts were excised. The ascending aorta was cannulated and perfused (120 mmHg) retrogradely with saline followed by Evans blue (1%). The right ventricle was then removed, and the left ventricle was cut into two slices. These slices were weighed and incubated in 1% triphenyltetrazolium chloride (TTC, Sigma; Poole, UK) in a pH 7.4 buffer during 15 min at 37°C to identify the infarcted myocardium. Videomicrographs were taken in 10% formaldehyde and then photographed with a digital camera. With the use of a computerized planimetric program (Scion Image, Scion; Frederick, MD), the area at risk and the infarcted zones were quantified. The area at risk was identified as the nonblue regions and was expressed as a percentage of the left ventricle weight. Infarcted area was identified as the TTC-negative zone and was expressed either as a percentage of the area at risk or as a percentage of the left ventricle weight.

Histological analysis. Formalin-fixed slices were further embedded in paraffin for histological analysis. Two 5-µm-thick sections were cut from each paraffin block using a microtome. One was stained with hematoxylin-eosin-safranin (HES) and the other with Masson’s trichrome. For morphometry, all HES-stained sections obtained from rabbits subjected to 72 h of CAR were observed in a microscope at a ×22 magnification. Successive digital photographs of the adjacent microscopic fields encompassing the complete cardiac circumference were recorded. A computerized reconstruction (Photoline, Computerinsel; Bad Gögging, Germany) of the complete cardiac slice at a ×22 magnification was made by adequate juxtaposition of the different and complementary digital photographs. Infarct was delimited from this photographic reconstruction by drawing its contour with a computer mouse. Simultaneous observation of the histologic section on a microscope at ×4 or ×10 magnifications allowed an accurate detection of the infarcted area. Myocardial infarction was considered as a central region of coagulation necrosis with a border of myocytolysis and inflammatory infiltration. Finally, planimetry was performed as previously described for the TTC-technique, and infarct sizes were calculated.

Data analysis. Data are reported as means ± SE. The effects of saline, CPA (100 or 400 µg/kg), and I-DPC on heart rate and mean arterial pressure were analyzed on day 1 by a paired Student t-test. On day 2, comparisons were made only between the four groups using ANOVA for repeated measurements. Comparisons were performed among control, I-DPC, A1-DPC100, and A1-DPC400 groups at each duration of CAR using a one-way ANOVA and post hoc Fisher’s protected least-significant difference test if necessary. Significant differences were determined as P < 0.05.

RESULTS

Sixty-one rabbits successfully underwent the CAO and subsequent reperfusion protocol and were used in this study: 10 in control, 7 in I-DPC, 6 in A1-DPC100, 6 in A1-DPC400 with 3 h of CAR and 12 in control, 6 in I-DPC, 8 in A1-DPC100, and 6 in A1-DPC400 with 72 h of CAR.

Hemodynamic. At day 1, baseline values of heart rate and mean arterial pressure were not significantly

Fig. 1. Experimental protocol. I-DPC, ischemia-induced delayed preconditioning; A1-DPC100, N6-cyclopentyladenosine (CPA)-induced delayed preconditioning, 100 µg/kg; A1-DPC400, CPA-induced delayed preconditioning, 400 µg/kg; CAO, coronary artery occlusion.
different among groups (heart rate: 214 ± 9, 205 ± 9, 202 ± 8, and 212 ± 10 beats/min; mean arterial pressure: 72 ± 3, 69 ± 3, 74 ± 3, and 70 ± 4 mmHg for control, I-DPC, A1-DPC100, and A1-DPC400, respectively). Intravenous injection of saline did not affect heart rate and mean arterial pressure in control (data not shown). In I-DPC, the ischemic preconditioning protocol induced a significant increase in heart rate during each CAO compared with baseline (e.g., +12 ± 4% during the last CAO), but mean arterial pressure did not change. In A1-DPC100 and A1-DPC400, CPA decreased both heart rate (−16 ± 3% and −20 ± 4%, respectively) and mean arterial pressure (−33 ± 3% and −39 ± 4%, respectively) compared with baseline. On day 2 (Table 1), heart rate and mean arterial pressure were not significantly different among control, I-DPC, A1-DPC100, and A1-DPC400 at baseline and during CAO and CAR.

Infarct sizes after 3 h of CAR. Left ventricular weights and area at risk sizes were similar among control, I-DPC, A1-DPC100, and A1-DPC400 with 3 h of CAR (Table 2). As illustrated in Fig. 2A, infarct sizes determined by TTC staining were similarly and significantly decreased in I-DPC, A1-DPC100, and A1-DPC400 (36 ± 5%, 41 ± 4%, and 38 ± 5% of the area at risk, respectively) compared with control (55 ± 3% of the area at risk). Because infarct size can be influenced by the size of the area at risk (28), the effects of I-DPC and A1-DPC were also investigated by plotting these two parameters expressed as percentages of the left ventricle. As shown in Fig. 2B, the infarct size/area at risk regression line was shifted downward by I-DPC, A1-DPC100, and A1-DPC400 compared with control. The overall histologic pattern of the heart sections obtained from rabbits subjected to 3 h of CAR was similar among the four groups. Infarcts consisted of myocyte necrosis with contraction bands and mainly of hemorrhages and edema. However, no clear-cut delimitation of the infarcted area was possible, and hence no accurate histologic determination of infarct size was possible after such a short CAR duration.

**Infarct sizes after 72 h of CAR.** Left ventricular weights and area at risk sizes were similar among control, I-DPC, A1-DPC100, and A1-DPC400 with 72 h of CAR (Table 2). As illustrated in Fig. 3A, infarct sizes

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**Table 1. Hemodynamics on day 2 in the different groups**

<table>
<thead>
<tr>
<th></th>
<th>Heart Rate, beats/min</th>
<th>Mean Arterial Pressure, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Baseline</td>
</tr>
<tr>
<td>Control</td>
<td>22</td>
<td>220 ± 7</td>
</tr>
<tr>
<td>I-DPC</td>
<td>13</td>
<td>211 ± 11</td>
</tr>
<tr>
<td>A1-DPC100</td>
<td>14</td>
<td>225 ± 8</td>
</tr>
<tr>
<td>A1-DPC400</td>
<td>12</td>
<td>232 ± 6</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of rabbits; CAO, coronary artery occlusion; CAR, coronary artery reperfusion; I-DPC, ischemia-induced delayed preconditioning; A1-DPC100 CPA-induced delayed preconditioning (100 μg/kg); A1-DPC400, CPA-induced delayed preconditioning (400 μg/kg).

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**Table 2. Left ventricular weight and area at risk in the different groups**

<table>
<thead>
<tr>
<th>Reperfusion 3 h</th>
<th>n</th>
<th>LV, g</th>
<th>AAR, %LV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>4.3 ± 0.1</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>I-DPC</td>
<td>7</td>
<td>4.1 ± 0.3</td>
<td>27 ± 4</td>
</tr>
<tr>
<td>A1-DPC100</td>
<td>6</td>
<td>4.2 ± 0.2</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>A1-DPC400</td>
<td>6</td>
<td>3.7 ± 0.3</td>
<td>26 ± 6</td>
</tr>
<tr>
<td>Reperfusion 72 h</td>
<td>12</td>
<td>4.7 ± 0.2</td>
<td>35 ± 5</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>4.0 ± 0.3</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>I-DPC</td>
<td>8</td>
<td>4.9 ± 0.2</td>
<td>32 ± 3</td>
</tr>
<tr>
<td>A1-DPC100</td>
<td>6</td>
<td>4.3 ± 0.3</td>
<td>29 ± 3</td>
</tr>
<tr>
<td>A1-DPC400</td>
<td>6</td>
<td>4.3 ± 0.3</td>
<td>29 ± 3</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; n, number of rabbits; LV, left ventricle; AAR, area at risk.

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**Fig. 2.** A: infarct sizes (expressed as percentage of area at risk) measured after 3 h of CAR. Infarction was detected after triphenyl-tetrazolium chloride (TTC) staining. Circles indicate individual and average values, respectively. B: scatterplots of the relationship between the sizes of the infarct and area at risk (expressed as percentage of left ventricular weight). Regression lines are represented for each group: control (r = 0.76), I-DPC (r = 0.79), A1-DPC100 (r = 0.79), and A1-DPC400 (r = 0.95). *P < 0.05 vs. control.
determined by TTC staining were not significantly different among control, I-DPC, A1-DPC100, and A1-DPC400 (41 ± 3, 42 ± 3, 40 ± 4, and 42 ± 3% of the area at risk, respectively). Furthermore, the infarct size/area at risk regression lines were not different among the four groups (Fig. 3B).

Importantly, as illustrated in Fig. 4A, infarct sizes determined by histology were also not significantly different among control, I-DPC, A1-DPC100, and A1-DPC400 (47 ± 4, 46 ± 3, 50 ± 4, and 46 ± 4% of the area at risk, respectively). The infarct size/area at risk regression lines were not different among the four groups (Fig. 4B). The overall histologic pattern of the heart sections obtained from all rabbits subjected to 72 h of CAR was similar among the four groups. Indeed, all infarcts consisted of a core of patent myocyte coagulation necrosis with some residual hemorrhage and edema. The infarcts were limited by a clear-cut border of detersion and huge inflammation.

In the control group, infarct sizes were not significantly different after 3 h of CAR and after 72 h of CAR by histology in agreement with Ytrehus et al. (28). The use of TTC at 72 h of CAR provided smaller values of infarct size due to a tendency for underestimation compared with histology (14).

**DISCUSSION**

In this study, we investigated the infarct-limiting effect of a pharmacological delayed preconditioning induced by two different doses of an adenosine A1-receptor agonist (CPA) in chronically instrumented conscious rabbits. In agreement with previous reports (2, 7, 8, 29), our results demonstrate that after 3 h of CAR, A1-DPC decreases infarct size. Similarly, I-DPC significantly decreased infarct size when assessed after 3 h of CAR, as also previously described in anesthetized (13) and conscious rabbits (14, 17). Surprisingly, both A1-DPC100 and A1-DPC400 failed to limit infarct size when measurements were performed after 72 h of CAR with both the TTC technique and the histologic analysis. Similarly, I-DPC failed to demonstrate any cardioprotective effect against myocardial infarction with 72 h of CAR.

One might argue that our negative results after 72 h of CAR are due to insufficient doses of CPA. However, this is unlikely because these doses were chosen on the basis of a dose-response curve as being the ED50 and the ED80 at decreasing mean arterial pressure, suggesting that the level of adenosine A1-receptor stimulation was high enough. Furthermore, the dose of 400 μg/kg was the highest hemodynamically well tolerated (26). Nevertheless and after 3 h of CAR, both doses of...
CPA elicited a significant reduction in infarct size and of the same magnitude, suggesting that the dose of 100 μg/kg was already sufficient to achieve a maximal cardioprotective effect. Importantly, the critical effect of the duration of reperfusion (i.e., 3 h vs. 72 h of CAR) was also observed with I-DPC in agreement with the data of Downey’s group (15). It is important to consider that the absence of cardioprotection afforded by A1-DPC and I-DPC after 72 h of CAR was observed regardless of the method used to measure infarct size (TTT technique and histologic analysis).

In apparent contradiction with the present results, Bolli’s group reported with similar experimental conditions (chronically instrumented conscious rabbits, TTC-technique), a significant limitation of infarct size after 72 h of CAR with I-DPC (17, 19, 22, 23) and with A1-DPC induced by 2-chloro-N6-cyclopentyladenosine (12, 21). This group further obtained a TTC-independent evidence for an infarct-limiting effect of I-DPC, i.e., an enhancement of the recovery of postinfarction myocardial function (24). Nevertheless, we also confirmed our results by a TTC-independent technique, i.e., histologic analysis. This discrepancy remains to be elucidated, but rabbits’ strain specificity might, at least in part, provide an explanation, as previously illustrated for I-DPC in mice hearts (1). Finally, we cannot rule out favorable remodeling effects of A1-DPC or I-DPC, which may enhance recovery of regional myocardial function in our experimental conditions.

The factors explaining the critical role of the duration of reperfusion on the cardioprotective effect of I-DPC and A1-DPC have not been investigated in the present study, but a number of hypotheses may be raised. According to most of the studies investigating infarct-limiting procedures, we defined myocardial infarction as being the TTC-unstained zone. TTC is a chemical that is converted to formazan dye by dehydrogenase enzymes and cofactors retained in the viable tissue (11). Tissue that is not stained by TTC, i.e., TTC-negative zone, is consequently considered as being infarcted. However, it is important to consider that tissue converting TTC into formazan could also be a dead tissue from which dehydrogenase enzymes have not yet been washed. Indeed, some interventions (e.g., intravenous superoxide dismutase administration) that could preserve capillary permeability and delay the dehydrogenases wash out might lead to a transient decrease of the TTC-negative zone without actual and prolonged cardioprotective effect (10, 20). Consequently, we cannot exclude that A1-DPC or I-DPC-induced infarct limitation after 3 h of CAR could be related to a delay in the loss of dehydrogenase enzymes in necrotic tissue secondary to a protection of the vasculature. Such a hypothesis, considered as unlikely by Downey’s group regarding I-DPC (15), has also been advocated to explain the inability of the free radical scavenger N-2-mercaptopyrrolidine to induce a prolonged limitation of infarct size (14). Finally, because tissue edema, hemorrhage, and acute inflammation are known to affect infarct size (18), one might suggest that the protection observed with A1-DPC and I-DPC after 3 h of CAR could be related to an attenuation of these phenomena. Indeed, tissue edema and hemorrhage, which may artificially increase infarct size after a short period of reperfusion, could be inhibited by delayed preconditioning, resulting in a decrease in infarct weight despite minor and no actual cardioprotective effect. Such a hypothesis is not further supported according to our qualitative analysis of infarction showing that huge hemorrhage and edema observed after 3 h of CAR are of similar extend in all groups of rabbits.

According to Birnbaum et al. (5), infarct size assessed by TTC following 30 min of myocardial ischemia, is smaller when measured after 2 h instead of after 4 h of CAR. It is reasonable to speculate that I-DPC and A1-DPC may delay the very early evolution of infarct size assessed by the TTC technique. This could explain the only transient protection observed with 3 h of CAR, whereas infarct size measured with 72 h of CAR is not significantly different from nonpreconditioned animals.

In conclusion, this study is the first to investigate in conscious rabbits the infarct-limiting effect of A1-DPC after both 3 and 72 h of CAR. A1-DPC, at the two CPA investigated doses, as well as I-DPC, significantly decreased infarct size after 3 h of CAR, but this effect was no longer observed after 72 h of CAR with both the TTC technique and histology.

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REFERENCES


