Cardiac effects of postconditioning depend critically on the duration of index ischemia

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Manintveld OC, te Lintel Hekkert M, van den Bos EJ, Suurenbroek GM, Dekkers DH, Verdouw PD, Lamers JM, Duncker DJ. Cardiac effects of postconditioning depend critically on the duration of index ischemia. Am J Physiol Heart Circ Physiol 292: H1551–H1560, 2007. First published November 22, 2006; doi:10.1152/ajpheart.00151.2006.—Postconditioning (POC) is known as the phenomenon whereby brief intermittent ischemia applied at the onset of reperfusion following index ischemia limits myocardial infarct size. Whereas there is evidence that the algorithm of the POC stimulus is an important determinant of the protective efficacy, the importance of the duration of index ischemia on the outcome of the effects of POC has received little attention. Pentobarbital sodium-anesthetized Wistar rats were therefore subjected to index ischemia produced by coronary artery occlusions (CAO) of varying duration (15–120 min) followed by reperfusion, without or with postconditioning produced by three cycles of 30-s reperfusion and reocclusion (3POC30). 3POC30 limited infarct size produced by 45-min CAO (CAO45) from 45 ± 3% to 31 ± 5%, and CAO60 from 60 ± 3% to 47 ± 6% (both P < 0.05). In contrast, 3POC30 increased infarct size produced by CAO15 from 3 ± 1% to 19 ± 6% and CAO30 from 36 ± 6 to 48 ± 4% (both P < 0.05). This deleterious effect of 3POC30 was not stimulus sensitive because postconditioning with 3POC5 and 3POC15 after CAO30 also increased infarct size. The cardioprotection by 3POC30 after CAO60 was accompanied by an increased stimulation of Akt phosphorylation at 7 min of reperfusion and a 36% lower superoxide production, measured by dihydroethidium fluorescence, after 2 h of reperfusion. Consistent with these results, cardioprotection by 3POC30 was abolished by phosphatidylinositol-3-OH-kinase inhibition, as well as nitric oxide (NO) synthase inhibition. The deleterious effect of 3POC30 after CAO15 was accompanied by an increased superoxide production with no change in Akt phosphorylation and was not affected by NO synthase inhibition. In conclusion, the effect of cardiac POC depends critically on the duration of the index ischemia and can be either beneficial or detrimental. These paradoxical effects of POC may be related to the divergent effects on Akt phosphorylation and superoxide production.

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RESTORATION OF BLOOD FLOW to ischemic myocardium is a prerequisite to interrupt the development of irreversible damage, but the mode of its application is of crucial importance. For instance, while gradual reperfusion may attenuate deleterious effects, abrupt reperfusion has been shown not only to increase the incidence of reperfusion arrhythmias (9) and aggravate stunning (16), but even to contribute to the development of irreversible damage (1, 5, 25, 26). Zhao et al. (39) reported recently that abrupt reperfusion after a sustained coronary artery occlusion (CAO) can also limit irreversible damage, provided that this was interrupted by a number of brief periods of abrupt coronary artery reocclusion and abrupt reperfusion started within 30 s after the initial abrupt reperfusion. The authors termed this phenomenon “postconditioning” (POC), because of its similarity to the multiple brief abrupt CAOs often employed in preconditioning protocols (39). Subsequent studies confirmed this original observation made in intact canine hearts in vivo and in situ rodent heart models (2, 7, 18, 19, 32, 38). These studies, which aimed to define the optimal algorithm of POC, also revealed that, to be effective, the first reocclusion has to be applied within 1 min after the onset of reperfusion (19), whereas increasing the number of reocclusion-reperfusion cycles beyond four cycles does not confer greater protection (18, 19, 38). Nevertheless, the optimal duration of the reocclusion and reperfusion periods of POC is currently unknown, but this most likely differs between animal species. Thus, although POC with three 30-s periods of abrupt CAO, starting 30 s after the initial reperfusion and interspersed by 30 s of abrupt reperfusion, limited infarct size produced by a 60-min CAO in the dog (13, 39), it failed to afford cardioprotection against a 30-min CAO in the rat (34). On the basis of this single observation, the authors suggested that briefer periods (i.e., 10–15 s) of reocclusion and reperfusion are required in smaller than in larger animals, in which 30 s cycles are effective (34), but conclusive evidence for this hypothesis is lacking. For instance, in discordance with this concept is a recent study by Schwartz and Lagrana (29), who showed in swine that three cycles of 30 s of reocclusion and reperfusion failed to limit infarct size produced by a 30-min CAO. Since POC with the 30-s algorithm was effective against a 60-min CAO (13, 39), but not against a 30-min CAO (29, 34), we hypothesized that the duration of the index ischemia also plays a major role in determining the effect of the POC stimulus. Consequently, the aim of the present study was to investigate the influence of index ischemia duration (ranging from 15 to 120 min) on the protective effect of POC. We subsequently investigated how the duration of index ischemia affected the role of potential mechanisms reported to be involved in the cardioprotection by POC such as the activation of the phosphatidylinositol-3-OH-kinase (PI3K)-Akt-endothelial nitric oxide (NO) synthase (eNOS) pathway and the production of reactive oxygen species (ROS) to explain our findings.
METHODS

Animals

Experiments were performed in male Wistar rats (300–380 g) in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and with prior approval of the Animal Care Committee of the Erasmus MC, University Medical Center Rotterdam.

Surgical and Experimental Procedures

Pentobarbital sodium-anesthetized (60 mg/kg ip) rats were intubated for positive pressure ventilation with oxygen-enriched room air. Through the carotid artery, a polyethylene (PE)-50 catheter was positioned in the thoracic aorta for measurement of arterial blood pressure and heart rate (10, 21–23, 33). In the inferior caval vein, a PE-50 catheter was placed for infusion of Gelofusin (5–10 ml; B. Braun Melsungen), to maintain central venous pressure at 4–6 mmHg, and for administration of drugs. After a thoracotomy was performed via the left third intercostal space, the pericardium was opened and a silk 6-0 suture was looped under the left anterior descending coronary artery for later occlusion of the vessel. A catheter was positioned in the abdominal cavity to allow intraperitoneal administration of pentobarbital for maintenance of anesthesia. Rectal temperature was maintained at 36.5–37.5°C (10, 22, 33).

Rats that fibrillated during occlusion or reperfusion were allowed to complete the protocol, provided that conversion to normal sinus rhythm occurred spontaneously within 1 min or that defibrillation via gently thumping on the thorax was successful within 2 min after onset of fibrillation. Occlusion and reperfusion were visually verified. The area at risk (AR) and infarct area (IA) were determined after 120 min.

Fig. 1. Overview of all experimental protocols in the present study. Protocols 1 and 2 pertain to the study of the effect of postconditioning (POC) on infarct size (IS), whereas protocols 3 and 4 pertain to the investigation of the potential mechanisms involved in postconditioning. In protocols 3 and 4 sham-operated animals, not undergoing ischemia, were included in the studies. CAOx denotes coronary occlusion durations ranging from 15 (CAO15) to 120 min (CAO120). 3POCx denotes different POC protocols, including 3 cycles of 5, 15, or 30 s. The arrow indicates the time of administration of drugs. eNOS, endothelial nitric oxide (NO) synthase.

Protocol 1: Importance of duration of index ischemia
1. CAOx
2. CAOx + 3POC30

Protocol 2: Importance of the postconditioning stimulus
3. CAO30
4. CAO30 + 3POC30
5. CAO60
6. CAO60 + 3POC30

Protocol 3: Involvement of PI3K/Akt-eNOS signaling pathway in postconditioning
7. CAO15
8. CAO15 + 3POC30
9. CAO60
10. CAO60 + 3POC30
11. CAO60
12. CAO60 + 3POC30

Protocol 4: Involvement of reactive oxygen species in postconditioning
13. CAO15
14. CAO15 + 3POC30
15. CAO60
16. CAO60 + 3POC30
17. CAO15
18. CAO15 + 3POC30
of reperfusion, using Trypan blue and Nitro-Blue-Tetrazolium staining, respectively (10, 22, 33). Infarct size (IS) was calculated as IA/AR × 100.

Experimental Protocols/Design

After completion of surgery, a 30-min stabilization period was allowed before animals were subjected to the experimental protocols (see Fig. 1 for overview of protocols). The duration of this stabilization period has been shown to be sufficiently long to exclude an effect of the surgical procedures on the development of infarct size (28).

Protocol 1: importance of the duration of index ischemia. Animals were subjected to periods of index ischemia consisting of a CAO varying between 15 min (CAO15) and 120 min (CAO120) in duration followed by abrupt reperfusion (control groups) or by postconditioning consisting of three cycles of 30 s of reperfusion and 30 s of reocclusion (3POC30) (13, 29, 39).

Protocol 2: importance of the POC stimulus. In another set of experiments, we investigated the influence of the POC stimulus. For this purpose, CAO30 and CAO60 were followed by a postconditioning stimulus consisting of three cycles of 5 s (3POC5) or three cycles of 15 s (3POC15) of reperfusion and reocclusion.

Protocol 3: involvement of PI3K-Akt-eNOS signaling pathway in POC. Four additional groups of rats were subjected to either 15 or 60 min of index ischemia followed by either abrupt reperfusion or 3POC30. After 7 min of abrupt reperfusion (control) or after postconditioning with 3POC30 followed by 7 min of reperfusion (3POC30) (7, 32), the AR was dissected and snap frozen in liquid nitrogen within 30 s before being stored at −80°C. The 7 min reperfusion time point was employed on the basis of previous POC studies (2, 4, 14, 15, 32) and in view of a previous in vitro study, in which Akt phosphorylation increased four- to fivefold within 10 min of reperfusion and remained elevated up to 60 min (24). Furthermore, phosphorylation (P) of both (Ser473)-Akt and (Thr308)-Akt has recently been shown to be obligatory for the full activation of Akt (36). Consequently, we determined P(Thr308)-Akt as well as P(Ser473)-Akt.

Additional rats were subjected to CAO60 with or without 3POC30 in the presence of the PI3K inhibitor wortmannin (15 µg/kg iv) (11) or the NO synthase inhibitor N•-nitro-l-arginine (l-NNA; 25 mg/kg iv) (23).

Protocol 4: involvement of ROS in POC. Four separate groups of animals were subjected to CAO15 or CAO60 followed by either abrupt reperfusion or 3POC30, while one group of control animals underwent only a sham procedure, i.e., without index ischemia. The four groups subjected to the 15-min or 60-min index ischemia were studied at the end of 120 min of reperfusion (39).

Two additional groups of rats underwent CAO15 either without or with 3POC30 (followed by 2 h of reperfusion), including treatment with the combination of ROS scavengers (N•-2-mercaptoethanol)-glycine (MPG; 30 mg•kg•h•1 iv), N-acetylcysteine (NAC; 250 mg•kg•h•1 iv), and Tempol (50 mg•kg•h•1 iv). Infusion was started 15 min before the onset of the CAO15 and was maintained throughout the experimental protocol. Starting 5 min before the onset of initial reperfusion, the infusion rates were doubled for 15 min.

Biochemical Assays

Akt phosphorylation. Akt phosphorylation was determined in the AR harvested at 7 min of reperfusion. Approximately 40 mg of frozen tissue were homogenized at liquid nitrogen temperature in a microdismembrator unit (B. Braun Biotech International). The frozen powder was suspended in 20 volumes of cold Laemmli loading buffer (20), and, thereafter, the suspensions were heated for 5 min at 95°C and sonicated in a Bioruptor. Protein determination was done using the RCDC protein assay (Bio-Rad). Proteins were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Immunoblot, Bio-Rad). Blots were preincubated in Tween-20 Tris-base sodium chloride [10 mmol/l Tris-HCl (pH 7.6), 150 mmol/l NaCl, and 0.1% Tween-20] supplemented with 5% BSA and incubated with diluted primary antibodies against Akt or phosphoThr308-Akt (rabbit polyclonal, Cell Signaling) or phosphoSer473-Akt (mouse monoclonal, New England Biolabs). Blots were probed with horseradish peroxidase-conjugated goat or mouse anti-rabbit secondary antibody. Signals were visualized using Supersignal West Femto Maximum Sensitivity Substrate (Pierce) and Hyperfilm enhanced chemiluminescence (ECL, Amersham Biosciences). Signal densities were quantified using a Bio-Rad calibrated GS-800 scanner.

Dihydroethidium fluorescence. Superoxide anion generation from ischemic-reperfused myocardium was determined by using dihydroethidium (DHE) fluorescence (27, 39). Hearts were excised and left ventricular transmural tissue samples were placed in ice-cold saline, embedded in Tissue-Tek (while marking the AR), frozen in liquid nitrogen, and stored at −60°C. Tissue sections of 5 µm were cut by using a cryostat, thaw mounted on Fisher-Plus (Fisher Scientific) slides, and stained with 10 µM DHE at 37°C for 30 min. Fluorescent images were obtained with a 585-nm long-pass filter. Generation of superoxide by tissue was demonstrated by red fluorescent labeling. Images were analyzed on a microscopy image analysis system (Impak C, Clemex vision image analysis system, Clemex Technologies, Longueuil, QC, Canada) on which a subroutine has been written to assess the total fluorescence per slide to quantify the amount of radical damage. At least five determinations were performed in each group.

Data Analysis and Presentation

Infarct size data were analyzed using two-way (duration of index ischemia × POC) ANOVA followed by post hoc testing with

![Fig. 2. Effect of postconditioning with 3POC30 on IS [percentage of area at risk (AR)] produced by CAOs of different duration. Open bars represent control infarcts; solid bars represent treatment with 3POC30. Data are means ± SE. Number of animals is shown below each bar.*P ≤ 0.05 vs. corresponding control; **P = 0.08 vs. corresponding control.](http://ajpheart.physiology.org/)

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Student-Newman-Keuls test. Heart rate and arterial blood pressure were analyzed using two-way (time × treatment) ANOVA for repeated measures followed by Student-Newman-Keuls test. SDS-PAGE allows loading of only 12 samples per gel. Therefore, the signal intensities on the immunoblot, expressed as fold increase compared with corresponding controls on the same gel, were analyzed using Student-Newman-Keuls test. Statistical significance was accepted when \( P \leq 0.05 \). All data are presented as means ± SE.

**RESULTS**

**Overall Mortality and Exclusion of Animals**

Of the 271 rats that entered the study, nine rats were excluded due to technical failure, five rats were excluded due to an AR <10% of the left ventricle, and four rats died prematurely during the index ischemia because of pump failure (no more than 1 rat per group).

**Importance of the Duration of Index Ischemia**

**Infarct size.** There were no intergroup differences in the AR (40 ± 1%; \( P = 0.56 \)) between the experimental groups (data not shown). Increasing the duration of index ischemia from 15 to 120 min produced a progressively greater infarct size in the animals of the control group, reaching a plateau after CAO60 (Fig. 2). Postconditioning with 3POC30 was cardioprotective when the stimulus was applied after CAO45 and CAO60, but it was ineffective when applied after coronary occlusions of longer duration (CAO90 and CAO120). Conversely, when the 3POC30 stimulus was applied following CAO15 and CAO30, infarct size was increased compared with their respective control groups (Fig. 2).

**Hemodynamics.** Baseline heart rate and mean arterial blood pressure for all animals allocated to the infarct size studies were 339 ± 3 beats/min and 93 ± 1 mmHg, with no significant differences in heart rate (\( P = 0.12 \)) and mean arterial blood pressure (\( P = 0.60 \)) between the experimental groups (Table 1). Collateral blood flow measurements were not performed in the present study, in view of earlier reports that the rat heart is devoid of a significant collateral circulation in the heart (12). Similar to previous observations from our laboratory (21), stepwise regression analysis (with occlusion duration, 3POC30, MAP, and 3POC30 × occlusion duration) revealed significant increases in heart rate and mean arterial blood pressure for all animals allocated to the infarct size studies were 339 ± 3 beats/min and 93 ± 1 mmHg, with no significant differences in heart rate (\( P = 0.12 \)) and mean arterial blood pressure (\( P = 0.60 \)) between the experimental groups (Table 1). Collateral blood flow measurements were not performed in the present study, in view of earlier reports that the rat heart is devoid of a significant collateral circulation in the heart (12). Similar to previous observations from our laboratory (21), stepwise regression analysis (with occlusion duration, 3POC30, MAP, and 3POC30 × occlusion duration) revealed significant increases in heart rate and mean arterial blood pressure.

**Table 1. Heart rate and arterial blood pressure of the rats studied for the effects of postconditioning with 3POC30 on infarct size**

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>n</th>
<th>Baseline</th>
<th>15-min CAO</th>
<th>End CAO</th>
<th>15 min</th>
<th>60 min</th>
<th>120 min</th>
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<td>CAO15 HR</td>
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<td>342 ± 17</td>
<td>348 ± 15</td>
<td>348 ± 15</td>
<td>368 ± 14</td>
<td>343 ± 14</td>
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<td>79 ± 7</td>
<td>80 ± 9</td>
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<td>CAO15 + POC HR</td>
<td>6</td>
<td>355 ± 10</td>
<td>363 ± 10</td>
<td>363 ± 10</td>
<td>356 ± 10</td>
<td>355 ± 8</td>
<td>371 ± 9</td>
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<td></td>
<td></td>
<td>101 ± 5</td>
<td>100 ± 6</td>
<td>100 ± 6</td>
<td>98 ± 6</td>
<td>103 ± 6</td>
<td>99 ± 4</td>
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<td>CAO30 HR</td>
<td>12</td>
<td>359 ± 12</td>
<td>375 ± 11</td>
<td>363 ± 9</td>
<td>368 ± 11</td>
<td>373 ± 15</td>
<td>374 ± 15</td>
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<tr>
<td></td>
<td></td>
<td>93 ± 4</td>
<td>92 ± 7</td>
<td>89 ± 4</td>
<td>91 ± 4</td>
<td>87 ± 6</td>
<td>79 ± 7</td>
</tr>
<tr>
<td>CAO30 + POC HR</td>
<td>9</td>
<td>337 ± 15</td>
<td>353 ± 14</td>
<td>349 ± 17</td>
<td>360 ± 15</td>
<td>366 ± 13</td>
<td>375 ± 13*</td>
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<td>88 ± 6</td>
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<td>357 ± 14</td>
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<td>326 ± 9</td>
<td>336 ± 11*</td>
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<td>342 ± 8*</td>
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<td>88 ± 3</td>
<td>83 ± 4</td>
<td>91 ± 6</td>
<td>87 ± 6</td>
<td>67 ± 7</td>
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</table>

Values are means ± SE. POC, postconditioning; CAO, coronary artery occlusion; HR, heart rate; MAP, mean arterial pressure. 3POC30, three cycles of 30 s of reperfusion and reocclusion. CAO15, -30, -45, -60, -90, and -120 refer to 15, 30, 45, 60, 90, and 120 min of CAO, respectively. *\( P < 0.05 \) vs. corresponding baseline.
stimulus, and rate-pressure product as independent variables) showed that there was no correlation between infarct size and the rate-pressure product either at the onset of index ischemia ($P = 0.61$) or at the onset of initial reperfusion ($P = 0.17$; data not shown).

**Importance of the POC Stimulus**

The additional experiments using a postconditioning stimulus with shorter reocclusion-reperfusion cycles revealed that the increase in infarct size by 3POC30 following CAO30 was not specific for this particular postconditioning stimulus, because infarct size was similarly increased when CAO30 was followed by 3POC5 ($P = 0.15$ vs. control) or 3POC15 ($P < 0.05$ vs. control; Fig. 3). Furthermore, there was no significant difference between the effects of the three different POC stimuli ($P > 0.20$). However, there was a significant difference compared with control when all three POC stimuli were combined compared with control ($P < 0.01$). Conversely, both 3POC5 ($P = 0.13$) and 3POC15 ($P = 0.18$) failed to emulate the protection against CAO60 that was observed with 3POC30 ($P < 0.05$). Also, in the CAO60 groups, there was no significant difference between the effects of the three different POC stimuli ($P > 0.20$), whereas there was a significant difference compared with control when all three POC stimuli were combined ($P < 0.05$).

**Involvement of PI3K-Akt-eNOS Signaling Pathways in POC**

**Akt phosphorylation.** Total Akt was unchanged at 7 min of reperfusion after CAO15 as well as CAO60. However, there were marked increments in P(Thr308)-Akt (10- and 7-fold, respectively) and P(Ser473)-Akt (8- and 4-fold, respectively) (Fig. 4, A and B). Postconditioning with 3POC30 following CAO60 produced further increase in P(Thr308)-Akt, but the further increase in P(Ser473)-Akt failed to reach statistical significance (Fig. 4, A and C); these increases were attenuated in the presence of the PI3K-inhibitor wortmannin (example shown in Fig. 4A). Conversely, following CAO15, 3POC30 did not increase but rather tended to decrease levels of P(Thr308)-Akt and P(Ser473)-Akt (Fig. 4C).

**Infarct size.** Both wortmannin and the NO synthase inhibitor l-NNA prevented the infarct size limitation by 3POC30 following CAO60 (Fig. 5). These observations suggest that the PI3K-Akt signaling pathway mediated the cardioprotection by 3POC30 via an increase in NO synthase activity.

**Hemodynamics.** Wortmannin had no effect on heart rate and mean arterial blood pressure (data not shown). l-NNA produced a $38 \pm 4$ mmHg increase in mean arterial pressure, which was accompanied by a 19 ± 3 beats/min decrease in heart rate (both $P \leq 0.05$).

**Involvement of ROS in POC**

**DHE fluorescence.** DHE reacts with superoxide anions to form ethidium bromide, which, in turn, intercalates with DNA to provide nuclear fluorescence as a marker for superoxide anion generation. As shown in Fig. 6, DHE fluorescence was markedly enhanced at 120 min of reperfusion following CAO60 compared with sham-operated animals (sham); 3POC30 attenuated the DHE fluorescence at 120 min of reperfusion following CAO60 by 36% (Fig. 6, C and D). DHE fluorescence was not affected at 120 min of reperfusion by CAO15 compared with sham, which is consistent with observations that the burst of ROS occurs principally during the first few minutes of reperfusion following CAO15 (3). Conversely, 3POC30 following CAO15 increased DHE fluorescence at 120 min of reperfusion by 12%, compared with CAO15 alone.

**Infarct size.** Treatment with a combination of ROS scavengers MPG-NAC-Tempol, which by itself had no effect on

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**Fig. 3. Effect of postconditioning with 3POC5, 3POC15, and 3POC30 on IS (percentage of AR) produced by CAO30 and CAO60.** Open bars represent control infarct; gray, hatched, and black bars represent treatment with 3POC5, 3POC15, or 3POC30, respectively. Data are means ± SE. *$P < 0.05$ vs. corresponding control; $P = $ NS indicates nonsignificance for comparison between the three POC stimuli.
infarct size produced by CAO15, abolished the increase in infarct size produced by 3POC30 after CAO15 (Fig. 7). Conversely, L-NNA had no significant effect on the increase of infarct size produced by 3POC30 after CAO15.

**Hemodynamics.** Treatment with a combination of ROS scavengers MPG-NAC-Tempol produced an 11 ± 4 mmHg decrease in mean arterial blood pressure (with no change in heart rate) 15 min after onset of the infusion, while an additional decrease in pressure of 18 ± 4 mmHg occurred (accompanied by a 42 ± 9 beats/min increase in heart rate) during the 15 min that the infusion rates were doubled (all P ≤ 0.05).

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**Fig. 4.** Effect of postconditioning with 3POC30 on Akt phosphorylation. A: representative examples of Western blots of Akt, phospho(Thr308)-Akt, and phospho(Ser473)-Akt for CAO15 and CAO60 in the absence and in the presence of postconditioning with 3POC30 and wortmannin. B: effect of CAO15 and CAO60 on the normalized average data of these blots. C: normalized average data for rats undergoing CAO15 or CAO60 without (open bars) or with (solid bars) 3POC30. Data are means ± SE; number of animals is shown in each bar. *P ≤ 0.05 vs. corresponding rats treated with sham procedure (sham); †P ≤ 0.05 POC vs. corresponding Control.
DISCUSSION

The present results show that postconditioning of the intact rat heart by 3POC30 after CAO45 and CAO60 limits infarct size but is ineffective when applied following CAO90 and CAO120, and it even aggravated infarct size when applied after CAO15 and CAO30. The detrimental effect of postconditioning on infarct size after these shorter periods of index ischemia was not stimulus specific because postconditioning after CAO30 with 3POC5 or 3POC15 resulted in similar infarct size as postconditioning with 3POC30. The modest degree of cardioprotection by 3POC30 (which was not afforded by 3POC5 or 3POC15) after CAO60 was accompanied by a further increase in stimulation of Akt phosphorylation and a reduced stimulation of superoxide production and was abolished by PI3K- as well as NO synthase inhibition. The increase in infarct size by 3POC30 after CAO15 was accompanied by an increase in superoxide production and a tendency toward a decreased stimulation of Akt phosphorylation. Also at variance with 3POC30 after 60-min CAO, NO synthase inhibition did not affect infarct size when 3POC30 was applied after the shorter periods of index ischemia. The implications of these results will be discussed.

Importance of Index Ischemia Duration

Currently, it is incompletely understood what the optimal duration of the reocclusion and reperfusion periods of POC is and to what extent its cardioprotective effect depends on the duration of index ischemia, experimental conditions or animal species (34). For example, while four 30-s periods of abrupt
CAO starting 30 s after the initial reperfusion, and interspersed by 30 s of abrupt reperfusion, limited infarct size produced by a 30-min CAO in the in situ rabbit heart (38), it failed to afford significant protection in the isolated buffer-perfused rabbit heart (37). Furthermore, while three 30-s periods of abrupt CAO starting 30 s after the initial reperfusion, and interspersed by 30 s of abrupt reperfusion, limited infarct size produced by a 60-min CAO in the dog (13, 39), it failed to afford cardioprotection against a 30-min CAO in the rat (34). The latter authors proposed that, in small rodents, briefer periods (i.e., 10–15 s) of reocclusion and reperfusion are required, whereas in larger animals, 30-s cycles are effective (34). In discordance with this notion, however, a recent study in swine showed that three cycles of 30 s of reocclusion and reperfusion failed to afford protection against infarct size produced by a 30-min CAO (29). Since the 30-s algorithm was effective against a 60-min CAO (13, 39), but not always against a 30-min CAO (29, 34, 37), we hypothesized that another important determinant of the optimal algorithm could be the duration of index ischemia.

The present study demonstrates that the effect of POC on myocardial infarct size in the rat heart depends critically on the duration of the index ischemia. Thus, while cardioprotection was observed with 3POC30 following the 45- and 60-min CAO, protection was lost with the longer occlusion durations of 90 and 120 min. This loss of protection likely reflects the progressive contribution of ischemic damage to infarction, with little contribution of reperfusion injury, when the duration of index ischemia is prolonged. Paradoxically, we observed that with 15-min CAO (which elicited negligible infarction under control conditions) as well as with 30-min CAO, 3POC30 aggravated irreversible damage. The increase in infarct size after postconditioning with 3POC30 was not a consequence of the application of this stimulus in a small animal model because postconditioning with 3POC5 or 3POC15, purportedly appropriate stimuli for small animals (34), also increased infarct size. Nevertheless, our data differ from that study (34), in which it was shown that postconditioning with either 3POC10 or 3POC15 after 30-min CAO limited infarct size, while postconditioning with 3POC30 had no effect (34). An explanation for these different observations is not readily found, but there are some differences between the two studies. First, although both studies employed male rats, we used Wistar rats, whereas Vinten-Johansen et al. (34) used Sprague-Dawley rats. Another difference in experimental design is the use of isoflurane in addition to the pentobarbital anesthesia in the study by Vinten-Johansen et al. (34). This could be important, because it has been reported that isoflurane can protect the myocardium against irreversible reperfusion damage at least, in part, by activation of PI3K-signaling (6, 8, 35). Nevertheless, our study indicates that the effect of POC not only depends on the algorithm of POC but also on the duration of index ischemia. The present data may also help to understand why, in pigs, postconditioning CAO30 with 3POC30 was not cardioprotective (29), while myocardial postconditioning in this species with the same stimulus after CAO75 was effective (17). Furthermore, it may help to explain why, in rats, in one study, 6POC10 afforded protection against CAO30 (34) but failed to protect against CAO60 (31).

**Involvement of PI3K-Akt-eNOS Signaling Pathway and ROS**

The novel finding that the effect of POC on infarct size could be double edged, i.e., beneficial or detrimental, depending on the duration of the index ischemia, warranted further investigation on the molecular mechanisms involved. There is already substantial evidence that several reperfusion injury survival kinase (RISK) pathways play a role in the cardioprotection by POC (34). For example, studies in the rabbit indicate that POC activates ERK1/2 (7), while the protection by POC is abolished by the ERK1/2 inhibitor PD-98059 (7, 38). On the other hand, the PI3K-Akt prosurvival pathway has been implicated in the protection in the isolated buffer-perfused rabbit (37) and rat (32) heart. The present study extends those observations to the in vivo rat heart and shows that POC following CAO60 not only further increases Akt-phosphorylation, but also that the reduction of infarct size is abolished by the selective PI3K inhibitor wortmannin (11). Further evidence for the involvement of the PI3K-Akt prosurvival pathway is the abolition of the POC-induced protection by the NO synthase inhibitor l-NNA, which corroborates previous findings in the in vivo rabbit heart (38). How the PI3K-Akt-eNOS pathway exerts its protection cannot be derived from the present observations, but there is evidence that preventing the opening of the mitochondrial permeability transition pore (mPTP) forms a crucial step in mediating the protection by POC (2, 4, 14, 15, 32). NO can inhibit opening of the mPTP directly but also indirectly by scavenging superoxide. In agreement with previous observations (19, 39), it was observed in the present study that 3POC30 attenuated the superoxide anion production at 120 min of reperfusion, which was likely due to an increased NO production by activation of the PI3K-Akt-eNOS pathway.

Paradoxically, 3POC30 following CAO15 increased infarct size. This was at variance with 3POC30 following CAO60, accompanied by an unchanged activity of the PI3K-Akt pro-survival pathway. Inhibition of NO synthase had no effect on...
the aggravation of irreversible injury produced by 3POC30, which is consistent with the lack of further increase of stimulation of the PI3K-Akt-eNOS prosurvival pathway. The apparent lack of NOS activation may also explain why we observed an increase, rather than a decrease, in superoxide anion production at 120 min of reperfusion when POC followed CAO15. Administration of a combination of ROS scavengers abolished the damage by 3POC30 after CAO15, indicating that the increased oxidative stress produced by the intermittent reoclusion and reperfusion sequences, following the shorter periods (15 and 30 min) of index ischemia, contributes to the increase in infarct size. Indeed, in the case of the 15-min index ischemia, only 3% infarct size is observed under control conditions, which increased to 19% when 3POC30 was applied. The mechanism by which oxidative stress aggravates necrosis under this condition cannot be derived from the present study and should be the subject of future investigations.

Conclusions

The effect of POC on myocardial infarct size is ambiguous. Although most studies, including one in humans (30), have shown that POC is cardioprotective (2, 7, 18, 19, 32, 38), there are several reports that have failed to observe a cardioprotective effect (29, 34, 37). This discrepancy has been ascribed to the distinct effects of POC which is consistent with the lack of further increase of stimulation of the PI3K-Akt-eNOS prosurvival pathway. The apparent lack of NOS activation may also explain why we observed an increase, rather than a decrease, in superoxide anion production at 120 min of reperfusion when POC followed CAO15. Administration of a combination of ROS scavengers abolished the damage by 3POC30 after CAO15, indicating that the increased oxidative stress produced by the intermittent reoclusion and reperfusion sequences, following the shorter periods (15 and 30 min) of index ischemia, contributes to the increase in infarct size. Indeed, in the case of the 15-min index ischemia, only 3% infarct size is observed under control conditions, which increased to 19% when 3POC30 was applied. The mechanism by which oxidative stress aggravates necrosis under this condition cannot be derived from the present study and should be the subject of future investigations.

GRANTS

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