Interaction Between Pre- and Postconditioning in the *In Vivo* Rat Heart

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Patients with an impending myocardial infarction may be preconditioned by pre-infarct angina. Hence, it is important to establish whether ischemic postconditioning is still effective in preconditioned hearts. We therefore studied in anesthetized rats the effect of postconditioning after coronary artery occlusions (CAO) of 60 min in control hearts, hearts preconditioned by a single 15-min CAO (1IPC15) or a triple 3-min CAO (3IPC3). Furthermore, we studied the effect of postconditioning in hearts that had been pharmacologically preconditioned with intravenous adenosine and in hearts that had become tolerant to 1IPC15. Postconditioning limited infarct size in control hearts, but did not afford additional protection in preconditioned hearts, irrespective of the IPC stimulus. NO synthase inhibition abolished the cardioprotection by postconditioning, both IPC stimuli, and the combination of postconditioning and either IPC stimulus. Postconditioning also failed to afford cardioprotection in hearts protected by adenosine, and in hearts that had become tolerant to cardioprotection by 1IPC15. In accordance with previous observations, postconditioning paradoxically increased infarct size following a 30-min CAO. This detrimental effect was prevented by either IPC stimulus, in a NO synthase–dependent manner. In conclusion, postconditioning does not afford additional protection in preconditioned hearts, irrespective of the preconditioning stimulus and the presence of tolerance to preconditioning. Lack of additional protection may be related to the observation that postconditioning and preconditioning are both mediated via NO synthase. In contrast, the increase in infarct size by postconditioning following a 30-min CAO is abolished by either IPC stimulus. These findings indicate that the interaction between preconditioning and postconditioning is highly dependent on the duration of index ischemia, but independent of the preconditioning stimulus. Exp Biol Med 234:1345–1354, 2009

Key words: adenosine; myocardial infarction; nitric oxide; preconditioning; postconditioning

Introduction

Postconditioning, i.e., multiple sequences of brief reocclusions during early reperfusion (stuttering reperfusion) (1) or gradual reperfusion (2–6) following a period of sustained ischemia, has been proposed as an adjunctive strategy to optimize reperfusion therapy in patients with acute myocardial infarction (7, 8). Although preliminary clinical trials in small groups of patients have reported encouraging results (9–12), it remains to be established whether postconditioning is effective in large, randomized clinical trials. Caution is also warranted in view of experimental studies in which postconditioning has been reported to exert not only protective or neutral effects (8, 13–15) but may even be detrimental (15, 16), which could depend on the postconditioning algorithm, duration of index ischemia, anesthesia regimen, and/or animal species/strain (8, 13–16). For example, we observed in the *in vivo* rat heart that although postconditioning was protective against infarction produced by 60 min of index ischemia produced by coronary artery occlusion (CAO60), it increased infarct size after 30 min (CAO30) of index ischemia (16).

Another issue that remains unresolved is whether preexisting myocardial ischemia modulates the effects of postconditioning on infarct size. This is an important issue, as many patients with an acute myocardial infarction may experience pre-infarct angina. Preliminary clinical trials have either excluded patients with pre-infarct angina (10, 12, 17) or did not report the occurrence of pre-infarct angina (9, 11), leaving the question principally unanswered. In contrast, the few experimental studies that have addressed this issue have reported ambiguous results (18–20), which could be due not only to the aforementioned factors

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(species, anesthesia, and postconditioning algorithm) (8), but also to the nature of the employed ischemic preconditioning (IPC) stimulus (21, 22) and the duration of index ischemia (16). For example, as stated above, depending on the duration of index ischemia, postconditioning can be protective, have no effect, or even be detrimental (15, 16). Furthermore, not all IPC stimuli employ the same signal transduction pathway to exert cardioprotection, as some stimuli employ an adenosine-dependent pathway, while others protect the myocardium via an adenosine-independent pathway (22). In light of these considerations, the first aim of the present study was to determine the interaction between preconditioning and postconditioning, taking into account the mechanism by which preconditioning stimuli exert cardioprotection. Additionally, in view of the proposed role of NO in the signal transduction of both IPC (23, 24) and postconditioning (19, 20, 25), we assessed the role of NO in the cardioprotection by IPC, postconditioning, and their combination.

Because of its involvement in the protection by ischemic preconditioning, adenosine has been applied in several experimental as well as clinical studies as a means to pharmacologically precondition the myocardium (26–29). It is therefore tempting to extrapolate the results of the interaction between an adenosine-dependent IPC stimulus and postconditioning to that of exogenously applied adenosine and postconditioning. However, we have previously shown that, in contrast to endogenously-released adenosine by ischemic preconditioning, intravenous infusion of exogenous adenosine does not increase myocardial interstitial levels and initiates cardioprotection, in part, also at remote extra-cardiac sites involving the activation of a neurogenic pathway similar to remote ischemic preconditioning (30). In view of these considerations, we also studied the effects of postconditioning in hearts preconditioned by intravenous adenosine.

Repeated bouts of a preconditioning stimulus may render the heart tolerant to the protective effects of ischemic preconditioning (31, 32). It is presently also unknown whether such preconditioning-resistant myocardium can still be protected by postconditioning. Consequently, the third aim of the present study was to determine whether, in myocardium that had become tolerant to preconditioning (31, 32), protection could be reinstated by postconditioning.

Recently, we observed that postconditioning, while protective after CAO60, paradoxically increased infarct size when applied after 30 min of index ischemia (16). Hence, the final aim of the present study was to determine whether preconditioning modulated the myocardial damage produced by postconditioning after CAO30.

Methods

Animals. Experiments were performed in 280 male Wistar rats (300–380 g) in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 86–23, revised 1996) and with prior approval of the Animal Care Committee of the Erasmus MC, University Medical Center Rotterdam.

Surgical and Experimental Procedures. Pentobarbital-anesthetized (60 mg/kg intraperitoneally) rats were intubated for positive pressure ventilation with oxygen-enriched room air. Through the carotid artery, a PE-50 catheter was positioned in the thoracic aorta for measurement of arterial blood pressure and heart rate (16, 32, 33). In the inferior caval vein, a PE-50 catheter was placed for infusion of Gelofusin (5–10 ml; B. Braun, Melsungen AG) to maintain central venous pressure at 4–6 mm Hg and for administration of drugs. After thoracotomy, 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 (16, 32, 33). After completion of surgery, a 30-min stabilization period was allowed before animals were subjected to the experimental protocols. 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 of reperfusion, using Trypan Blue and Nitro-Blue-Tetrazolium staining, respectively (16, 32, 33). Infarct size was calculated as IA/AR×100%.

Experimental Protocols. In the first series of experiments, we investigated whether postconditioning can afford additional protection in preconditioned myocardium and whether this effect depends on the mechanism by which the myocardium is preconditioned. For this purpose, we subjected rats to a 60-min coronary artery occlusion (CAO60) followed by 120 min of reperfusion. Rats underwent a control period or were preconditioned with either a 15-min coronary artery occlusion preceding the index ischemia by 10 min reperfusion (1IPC15) or 3 sequences of 3-min coronary artery occlusion and 5 min of reperfusion (3IPC3) (Fig. 1, Protocol 1). These 2 preconditioning stimuli were used, as they employ markedly different signaling pathways, i.e., 1IPC15 is adenosine-dependent, but reactive oxygen species (ROS)–independent, whereas 3IPC3 is ROS-dependent, but adenosine-independent (21, 22). During early reperfusion, rats underwent either a sham period or postconditioning with 3 cycles of 30s coronary artery occlusion separated by 30s of reperfusion, starting 30s after reperfusion (3POC30). The 3POC30 algorithm was chosen, as we have previously shown that it limits infarct size in the *in vivo* rat heart and is equally effective in reducing infarct size compared to 3 cycles of either 5s or 15s (16). Since NO plays a critical role in the

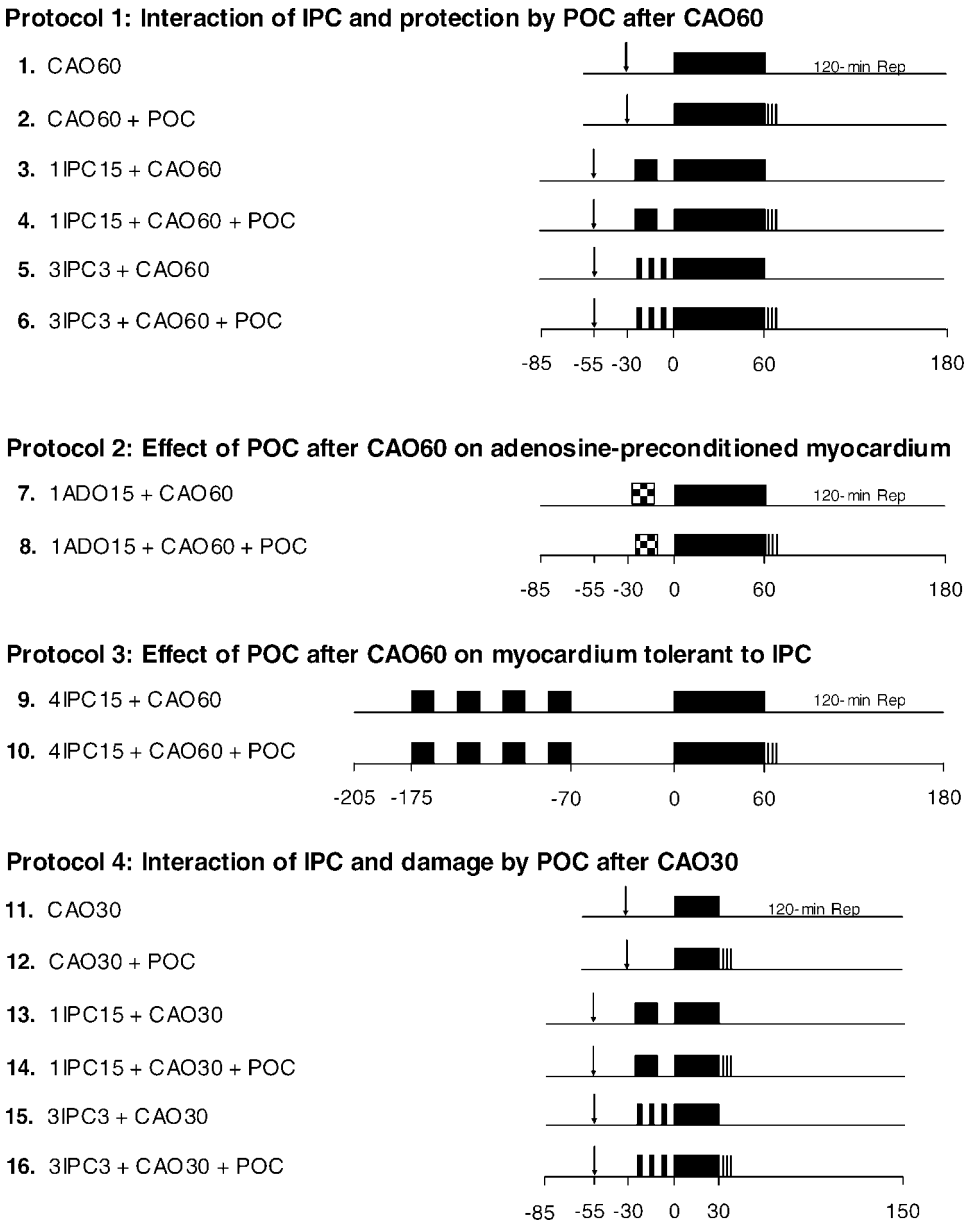

Figure. 1. Shown are the infarct size protocols involving a 60-min CAO or a 30-min CAO with the different preconditioning stimuli (1IPC15, 3IPC3, or 1ADO15) with or without 3POC30 including the administration of LNNA (↓).

cardioprotection by postconditioning (19, 20, 25), we investigated whether NO also plays a role in the interaction between the protective effects of preconditioning and postconditioning. To this end, experiments were repeated in the presence of the NO synthase inhibitor N-nitro-L-arginine (LNNA, 25 mg/kg iv) (16, 30).

In a second series of experiments, the effects of postconditioning in hearts preconditioned by a 15-min intravenous infusion of adenosine (200 µg/kg/min) followed by 10 min of washout (1ADO15; Fig. 1, Protocol 2) (30, 32) were evaluated.

In a third series of experiments, the effect of postconditioning on myocardium that had become tolerant to preconditioning was studied (Fig. 1, Protocol 3). Tolerance to preconditioning was induced by 4IPC15 interspersed by 15 min of reperfusion and applied between 175 and 70 min prior to the 60-min index ischemia (Fig. 1, Protocol 3) (32). Due to technical problems (see Discussion), we were unable to perform studies in rats that had become tolerant to multiple cycles of 3IPC3.

In view of recent observations, by us (16) and others (15), that the nPOC30 algorithm increases infarct size in the *in vivo* rat heart when applied following a brief period of index ischemia, we investigated in a fourth series of experiments whether preconditioning could protect against the detrimental effect of postconditioning on infarct size produced by a 30-min coronary artery occlusion (CAO30) and determined also in this model the role of NO in the

Table 1. Heart Rate and Arterial Blood Pressure[a]

Experimental groups	*n*	Baseline	Pre-CAO	End-CAO	15-min CR	60-min CR	120-min CR
Untreated groups							
CAO60 control	11						
HR		320 ± 5	313 ± 7	319 ± 6	320 ± 6	321 ± 5	335 ± 6*
MAP		94 ± 3	92 ± 3	89 ± 2	88 ± 2	88 ± 3	89 ± 4
1IPC15+CAO60	9						
HR		329 ± 5	328 ± 15	337 ± 15	337 ± 15	360 ± 17	350 ± 19
MAP		94 ± 5	87 ± 4	83 ± 5	85 ± 6	82 ± 6	74 ± 7*
3IPC3+CAO60	10						
HR		330 ± 16	334 ± 18	343 ± 21	341 ± 22	339 ± 24	346 ± 17
MAP		83 ± 4	90 ± 5	83 ± 6	87 ± 6	83 ± 6	72 ± 7*
CAO60+POC	12						
HR		327 ± 7	327 ± 7	342 ± 7*	342 ± 6*	355 ± 8*	365 ± 11*
MAP		94 ± 4	95 ± 3	99 ± 4	97 ± 4	93 ± 5	92 ± 5
1IPC15+CAO60+POC	10						
HR		341 ± 8	351 ± 7	354 ± 11	364 ± 11	373 ± 11*	395 ± 12*
MAP		92 ± 3	88 ± 5	90 ± 6	91 ± 7	84 ± 8	85 ± 5
3IPC3+CAO60+POC	8						
HR		348 ± 11	356 ± 14	366 ± 12	369 ± 12	377 ± 11*	396 ± 10*
MAP		94 ± 4	99 ± 4	94 ± 2	99 ± 3	93 ± 4	87 ± 4
LNNA-treated groups							
CAO60 control	10						
HR		315 ± 6	294 ± 7	313 ± 11	310 ± 10	318 ± 12	308 ± 17
MAP		88 ± 3	136 ± 4*	101 ± 5	84 ± 7	75 ± 6	70 ± 6*
1IPC15+CAO60	11						
HR		322 ± 10	295 ± 10*	298 ± 11*	297 ± 13*	310 ± 15	303 ± 7*
MAP		90 ± 3	114 ± 10*	109 ± 7*	104 ± 8	94 ± 8	71 ± 8*
3IPC3+CAO60	8						
HR		307 ± 13	265 ± 17*	287 ± 21	293 ± 19	308 ± 18	321 ± 19
MAP		81 ± 2	122 ± 9*	95 ± 8	87 ± 8	75 ± 7	67 ± 9
CAO60+POC	10						
HR		335 ± 7	302 ± 5*	305 ± 7*	302 ± 5*	303 ± 6*	320 ± 5
MAP		95 ± 5	121 ± 7*	97 ± 5	88 ± 4	85 ± 5	79 ± 7
1IPC15+CAO60+POC	9						
HR		321 ± 10	291 ± 8*	293 ± 8*	296 ± 7*	296 ± 7*	321 ± 11
MAP		86 ± 2	100 ± 4*	88 ± 4	85 ± 6	76 ± 8	80 ± 7
3IPC3+CAO60+POC	6						
HR		307 ± 6	266 ± 2*	264 ± 5*	269 ± 6*	283 ± 6*	285 ± 8*
MAP		86 ± 4	113 ± 3*	98 ± 2	97 ± 3	87 ± 3	66 ± 8*

HR, heart rate; MAP, mean aortic pressure; CAO, coronary artery occlusion; CR, coronary reperfusion.
[a] Data are mean ± SEM.
* $P < 0.05$ vs corresponding baseline.

interaction between preconditioning and postconditioning (Fig. 1, Protocol 4).

Data Analysis and Presentation. All data were analyzed using ANOVA for repeated (hemodynamics) or non-repeated (infarct size) measures, followed by *post hoc* testing with Student-Newman-Keuls test. Statistical significance was accepted when $P < 0.05$. All data are presented as mean ± SEM.

Results

Mortality and Exclusions. Of the 280 rats that entered the study, 3 rats were excluded due to technical failure, 6 rats were excluded due to acute heart failure upon reperfusion (no more than 2 rats in a single experimental group), while 5 rats were excluded due to an AR <15% of the left ventricle.

Hemodynamic Data. There were no significant differences in heart rate or mean aortic pressure at baseline between any of the treated groups undergoing CAO60 (Table 1), and only minor differences in the groups undergoing CAO30 (Table 2). LNNA produced an increase in blood pressure from 87 ± 1 to 110 ± 2 mm Hg, which was accompanied by a decrease in heart rate from 320 ± 2 to 289 ± 2 bpm (both $P < 0.05$). These changes were sustained until the onset of CAO60. 1ADO15 produced a decrease in blood pressure from 94 ± 2 to 59 ± 2 mm Hg, which was accompanied by a decrease in heart rate from 353 ± 6 to 334 ± 4 bpm (both $P < 0.05$). After discontinuation of adenosine, both heart rate and blood pressure recovered to baseline values well before the onset of CAO60. Importantly, there was no significant correlation between the rate-pressure product at the onset of the CAO

Table 2. Heart Rate and Arterial Blood Pressure[a]

Experimental groups	*n*	Baseline	Pre-CAO	End-CAO	15-min CR	60-min CR	120-min CR
Untreated groups							
CAO30 control	11						
HR		344 ± 12	348 ± 14	349 ± 14	355 ± 15	357 ± 17	362 ± 17
MAP		83 ± 3	89 ± 3	85 ± 3	90 ± 3	87 ± 4	78 ± 6
1IPC15+CAO30	11						
HR		359 ± 12*	352 ± 13	354 ± 12	353 ± 15	345 ± 13	357 ± 13
MAP		90 ± 2	87 ± 3	88 ± 4	91 ± 4	91 ± 3	91 ± 5
3IPC3+CAO30	8						
HR		343 ± 7	337 ± 8	348 ± 8	338 ± 7	340 ± 4	349 ± 7
MAP		100 ± 4*	98 ± 4	97 ± 2	98 ± 3	99 ± 3	93 ± 8
CAO30+POC	11						
HR		335 ± 9	333 ± 12	343 ± 14	352 ± 14†	354 ± 11†	367 ± 12†
MAP		92 ± 4	89 ± 5	86 ± 3	88 ± 4	87 ± 5	79 ± 4†
1IPC15+CAO30+POC	11						
HR		345 ± 9	355 ± 11	355 ± 11	344 ± 10	344 ± 7	354 ± 9
MAP		93 ± 4	93 ± 3	91 ± 4	95 ± 2	92 ± 3	90 ± 4
3IPC3+CAO30+POC	7						
HR		346 ± 9	347 ± 8	342 ± 8	341 ± 7	335 ± 8	346 ± 7
MAP		98 ± 3*	104 ± 3	92 ± 4	98 ± 4	96 ± 2	98 ± 3
LNNA-treated groups							
CAO30 control	8						
HR		329 ± 8	298 ± 4†	305 ± 5†	306 ± 5†	317 ± 11	316 ± 4
MAP		93 ± 5	113 ± 3†	103 ± 5	98 ± 5	93 ± 4	85 ± 5
1IPC15+CAO30	11						
HR		326 ± 7	299 ± 5†	295 ± 5†	297 ± 5†	318 ± 10	317 ± 6
MAP		85 ± 2	93 ± 5†	85 ± 4	86 ± 6	83 ± 5	72 ± 7†
3IPC3+CAO30	8						
HR		308 ± 7	275 ± 7†	279 ± 5†	280 ± 6†	283 ± 12†	279 ± 10†
MAP		78 ± 2	88 ± 5†	80 ± 5	77 ± 3	61 ± 5†	53 ± 4†
CAO30+POC	9						
HR		323 ± 4	293 ± 6†	297 ± 6†	295 ± 6†	302 ± 6†	313 ± 9
MAP		90 ± 4	114 ± 8†	99 ± 7	93 ± 7	93 ± 5	79 ± 8
1IPC15+CAO30+POC	9						
HR		322 ± 7	298 ± 5†	297 ± 5†	299 ± 5†	303 ± 5†	314 ± 8
MAP		85 ± 3	99 ± 6†	97 ± 4†	95 ± 4	93 ± 5	88 ± 6
3IPC3+CAO30+POC	6						
HR		317 ± 7	268 ± 6†	263 ± 9†	267 ± 8†	276 ± 6†	288 ± 11†
MAP		84 ± 3	110 ± 6†	91 ± 6	95 ± 7	79 ± 7	64 ± 6†

HR, heart rate; MAP, mean aortic pressure.
[a] Data are mean ± SEM.
* $P < 0.05$ vs baseline in 3IPC3+CAO30 pre-treated with LNNA.
† $P < 0.05$ vs corresponding baseline.

and the corresponding IS (linear regression: $r^2 = 0.007$; $P = 0.22$).

Infarct Size. There were no inter-group differences in area at risk (41 ± 1% of the left ventricle; $P = 0.46$) between the experimental groups.

In the first series, we investigated whether postconditioning and preconditioning afford additive cardioprotection. CAO60 resulted in an infarct size of 60 ± 3% of the area at risk. Preconditioning by 1IPC15 limited infarct size to 44 ± 5% and by 3IPC3 to 43 ± 3% (both $P < 0.05$ vs CAO60; Fig. 2); postconditioning with 3POC30 was equally cardioprotective (infarct size = 46 ± 5%, $P < 0.05$ vs CAO60). Postconditioning of myocardium already preconditioned by either 1IPC15 or 3IPC3 did not afford additional protection over that produced by preconditioning alone, irrespective of the IPC stimulus. LNNA had no effect on infarct size itself, but abolished not only the cardioprotection by 1IPC15, 3IPC3, and 3POC30 alone, but also by the combinations 1IPC15 + 3POC30 and 3IPC3 + 3POC30 (Fig. 2).

In the second series of experiments, we found that postconditioning of adenosine-preconditioned hearts did not further increase cardioprotection, since 1ADO15 followed by CAO60 ($n = 15$) resulted in an infarct size (42 ± 3%) that was similar to that (43 ± 6%) observed after 1ADO15 followed by CAO60 and postconditioning ($n = 9$).

In the third series of experiments, we investigated the effect of postconditioning with 3POC30 on myocardium that had become tolerant to the protection by preconditioning with 1IPC15 (32). Postconditioning after CAO60 of this

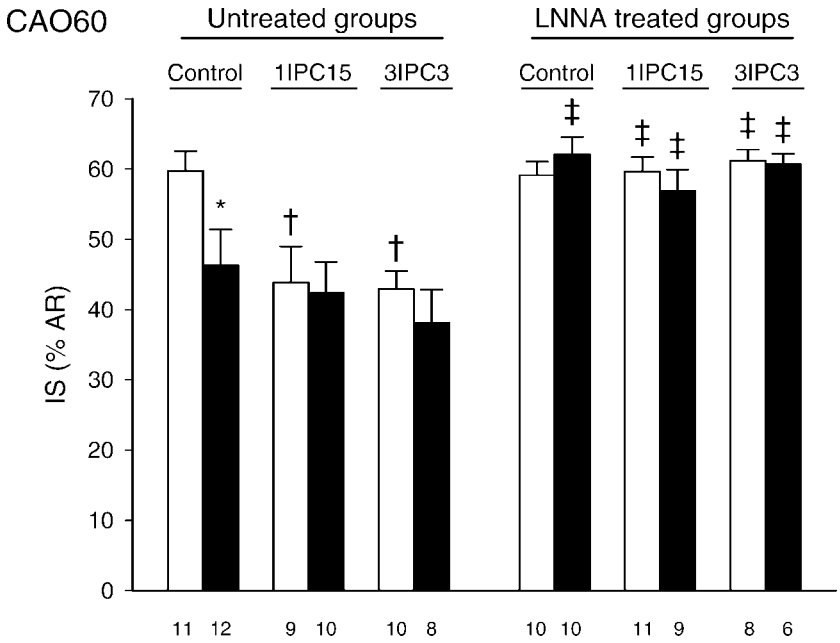

Figure 2. Effects of 3POC30 on myocardial infarct size produced by CAO60 in control hearts or hearts preconditioned by either 1IPC15 or 3IPC3. Postconditioning data are represented by black bars; sham data are represented by white bars. The number of animals is indicated below each bar. Data are mean ± SEM. * $P < 0.05$ vs corresponding Sham; † $P < 0.05$ vs corresponding Control; ‡ $P < 0.05$ vs corresponding untreated group.

preconditioning-tolerant myocardium resulted in an infarct size of 59 ± 3% ($n = 8$), which was not different from infarct size in rats undergoing only CAO60 (60 ± 3%) or in rats having undergone 4IPC15 followed by CAO60 ($n = 9$; 67 ± 3%), indicating that postconditioning failed to protect myocardium tolerant to preconditioning.

In the final series of experiments, we found that preconditioning with 1IPC15 or 3IPC3 before CAO30 limited IS to 21 ± 3% and 19 ± 4%, respectively, compared to 38 ± 3% in rats that underwent only CAO30 (both $P <$ 0.05; Fig. 3). However, postconditioning after CAO30 increased infarct size to 50 ± 3% ($P < 0.05$ vs CAO30). Preconditioning prevented the 3POC30-induced increase in infarct size after CAO30, irrespective of the employed IPC stimulus. LNNA, which did not affect infarct size after CAO30 by itself, abolished the cardioprotective effect of 1IPC15 and that of 3IPC3, as well as the protection by the combination of either preconditioning stimulus with 3POC30 (Fig. 3).

Discussion

The main findings of the present study in the *in vivo* rat heart are: (*i*) Postconditioning did not afford additional protection when hearts had been subjected to preconditioning prior to the CAO60, irrespective of the preconditioning stimulus (1IPC15 or 3IPC3 or 1ADO15). (*ii*) Postconditioning after CAO60 was unable to protect myocardium that had become tolerant to the protection by 1IPC15. (*iii*) Inhibition of NO synthase abolished the cardioprotection by postconditioning, IPC, and the combination of IPC + postconditioning after CAO60, irrespective of the IPC stimulus. (*iv*) Irrespective of the algorithm used, IPC prevented the increase in infarct size produced by postconditioning following CAO30. (*v*) Inhibition of NO synthase abolished this protection by preconditioning.

Effects of Postconditioning in Hearts Protected by Ischemic Preconditioning. The answer to the question whether postconditioning can further limit infarct size when myocardium is already preconditioned is ambiguous, as experimental studies (18–20) show either no additional (18, 19) or additional cardioprotection (20) when preconditioning and postconditioning were combined (Table 3). These divergent findings could be due to a number of factors, including animal species (13, 34), experimental conditions (35), algorithm of the postconditioning stimulus (8), the type of preconditioning stimulus employed (21, 22), and the duration of index ischemia (15, 16). Indeed, all 3 studies (18–20) were performed in different animal species, employing different signaling pathways (34, 36), and under different experimental conditions, which may influence the degree and mechanism of protection by preconditioning (37, 38) and postconditioning (35). Furthermore, all 3 studies differed in terms of algorithm of the postconditioning stimulus, period of index ischemia, and preconditioning stimulus (Table 3). We have previously shown that ischemic preconditioning stimuli can activate distinctly different signaling pathways (21, 22). For example, 1IPC15 is adenosine-dependent and ROS-independent, whereas 3IPC3 exerts its protective effect through ROS formation, but does not require adenosine receptor activation (21, 22). Interestingly, both stimuli affect mitochondrial respiration (22), which appears to be

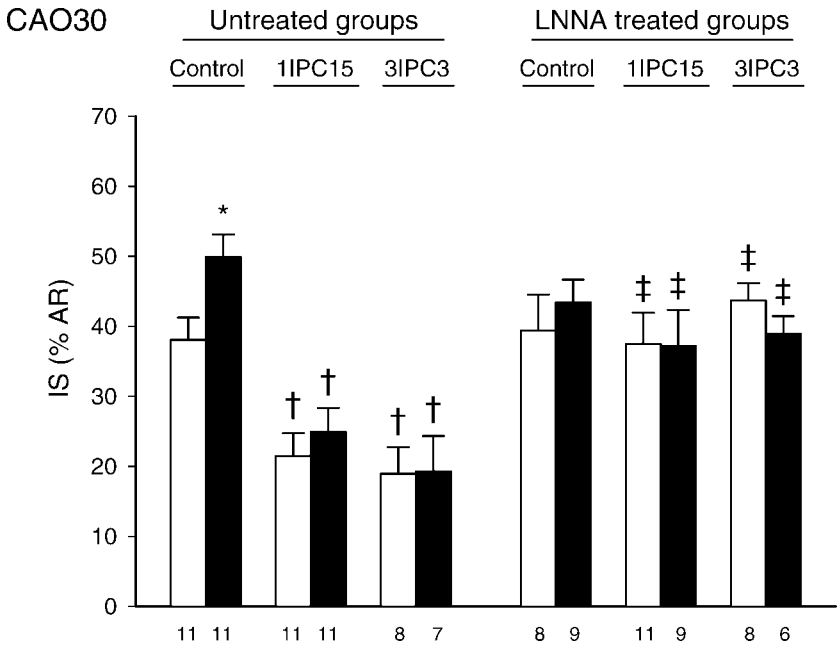

Figure 3. Effects of 3POC30 on myocardial infarct size produced by CAO30 in control hearts or hearts preconditioned by either 1IPC15 or 3IPC3. Postconditioning data are represented by black bars; sham data are represented by white bars. The number of animals is indicated below each bar. Data are mean ± SEM. * $P < 0.05$ vs corresponding Sham; † $P < 0.05$ vs corresponding Control; ‡ $P < 0.05$ vs corresponding untreated group.

mediated by NO (unpublished data from our laboratory), suggesting that these pathways ultimately converge at the level of NO-mediated modulation of mitochondrial respiration (39, 40). In light of these considerations, we specifically addressed whether and how postconditioning affects the development of infarct size of myocardium preconditioned by stimuli, employing different signal transduction pathways. We showed that postconditioning did not afford additional cardioprotection after CAO60 in the *in vivo* rat heart subjected to preconditioning, irrespective of the transduction pathway employed by the preconditioning stimulus. Although there is evidence for NO synthase–independent pathways in NO synthesis during myocardial ischemia (41), the observation that inhibition of NO synthase abolished the cardioprotection by either preconditioning stimulus and by postconditioning, either alone or in combination, suggests that both preconditioning and postconditioning stimuli require unperturbed NO synthase activity in order to exert their cardioprotection. Furthermore, these data could be interpreted to suggest that preconditioning already resulted in (near) maximum NO signaling so that postconditioning could not stimulate this pathway further. Future studies are required to investigate in more detail the molecular basis for the lack of additive effects of postconditioning to either preconditioning stimulus.

Although the present study fails to support the concept that postconditioning affords additional protection when myocardium has been preconditioned prior to the index ischemia, it could be argued that a different postconditioning algorithm (employing perhaps a different signaling pathway than preconditioning) might still be able to further limit infarct size in preconditioned myocardium (8, 36). Indeed, in the rabbit heart in which postconditioning (by 4 cycles of 30-s reperfusion and 30-s ischemia) and preconditioning (by 1IPC5) afforded additive cardioprotection, postconditioning activated extracellular signal–regulated kinase (ERK) without activating Akt (20, 42), while preconditioning was ERK-independent (20). Conversely, we have previously shown that the postconditioning algorithm that was used in the present study resulted in activation of Akt following a CAO60 in rats, while its cardioprotection was blocked by wortmannin (16), implicating a critical role for PI3K-Akt signaling. Interestingly, recent unpublished observations from our laboratory indicate that preconditioning by 1IPC15, but not 3IPC3, is associated with activation of Akt and is blocked by wortmannin (Manintveld *et al.*, unpublished data). The observation that the PI3K-Akt–dependent postconditioning algorithm did not afford additional protection in myocardium preconditioned with the PI3K-Akt–independent 3IPC3 stimulus suggests that other postconditioning algorithms, such as 3POC5 and 3POC15, which we have shown to be equally effective as 3POC30 in the *in vivo* rat heart (16), may also be unable to enhance protection in preconditioned rat hearts.

We have recently shown that while postconditioning afforded cardioprotection following CAO60, it increased infarct size when applied following CAO30 in the *in vivo* rat heart (16). This detrimental effect on infarct size was of similar magnitude when 3POC5 or 3POC30 were used,

Table 3. Studies on the Interaction Between POC and IPC Against Infarction Produced by Regional Myocardial Ischemia

Studies	Experimental set-up: Animal	Experimental set-up: Experimental conditions	IPC stimulus (I′+R′)	Index ischemia (I′+R′)	POC stimulus (R″+I″)	Infarct size (% of area at risk): Control	POC	IPC	IPC +POC
Halkos *et al.* (18)	Dog	Morphine chloralose anesthesia, open-chest	1× 5′+10′	60′+180′	3× 30″+30″	24 ± 2	10 ± 1*	13 ± 2*	12 ± 3*
Yang *et al.* (20)	Rabbit	Pentobarbital anesthesia, open-chest	1× 5′+10′	45′+180′	4× 30″+30″	62 ± 2	40 ± 4*	35 ± 4*	23 ± 3*,†
Tsang *et al.* (19)	Rat	Isolated buffer-perfused	2× 5′+10′	35′+120′	6× 10″+10″	51 ± 3	32 ± 4*	28 ± 2*	30 ± 5*
Manintveld *et al.*	Rat	Pentobarbital anesthesia, open-chest	3× 3′+ 5′	60′+120′	3× 30″+30″	60 ± 3	46 ± 5*	46 ± 2*	38 ± 5*
			1× 15′+10′	60′+120′	3× 30″+30″			44 ± 2*	42 ± 4*
			3× 3′+5′	30′+120′	3× 30″+30″	38 ± 3	50 ± 3*	19 ± 4*	19 ± 5*
			1× 15′+10′	30′+120′	3× 30″+30″			21 ± 3*	25 ± 3*

IPC, ischemic preconditioning; POC, postconditioning; I, ischemia; R, reperfusion.
* $P < 0.05$ vs Control.
† $P < 0.05$ IPC+POC vs IPC.

suggesting that it occurred independently of the postconditioning algorithm used (16). Furthermore, we observed that the increase in infarct size by postconditioning was prevented by anti-oxidant treatment but remained unaffected in the presence of NO synthase inhibition with LNNA (16). Those previous observations indicate that the cardioprotection by postconditioning following a longer index ischemia period involves a markedly different signaling pathway (involving NO) than that which mediates the detrimental effects of postconditioning following brief index ischemia (involving reactive oxygen species). Consequently, we investigated whether IPC (which also acts through NO) could protect the heart against the damage produced by 3POC30. The results of the present study show that IPC protected against the detrimental effects of postconditioning following CAO30 in the rat heart, which was indeed mediated by NO and was independent of the IPC stimulus. Future studies are required to investigate whether the detrimental effects of postconditioning following brief periods of index ischemia is a phenomenon exclusively observed in rats (15, 16) or that it occurs in other species as well. Furthermore, it is clear that future studies should investigate the molecular basis for the protective effect of preconditioning against the damage by postconditioning in the rat heart in more detail. Nevertheless, the observation that both IPC stimuli protected against the myocardial damage produced by postconditioning following CAO30, in conjunction with the finding that protection by postconditioning following CAO60 was lost when hearts had undergone IPC, is consistent with the concept that preconditioning exerts its protection principally during early reperfusion and that preconditioning shares a common pathway with the protective (but not the detrimental) effects of postconditioning (36, 43, 44).

Effects of Postconditioning in Myocardium Preconditioned by Adenosine Infusion. Adenosine has been shown to be a trigger of preconditioning in all animal species studied (21, 45). Interestingly, we have shown that preconditioning produced by intravenous adenosine infusion is not associated with a detectable increase in myocardial interstitial adenosine concentrations and acts, at least in part, via (remote) activation of a neurogenic pathway (30). Thus, the ganglion-blocker hexamethonium attenuated the cardioprotection by intravenous adenosine by as much as 65% (30), while it had no effect on the protection by classical preconditioning with 1IPC15 (33). Although the initiation by exogenous adenosine of its cardioprotective signaling pathway is markedly different from that employed by IPC, the cardioprotection by adenosine (30) and either IPC stimulus (present study) is critically dependent on NO synthase activation. The involvement of NO synthase in both postconditioning and intravenous adenosine may explain why we also did not observe additional protection by postconditioning in rats pretreated with intravenous adenosine.

Efficacy of Postconditioning in Myocardium Made Tolerant to Preconditioning. Myocardium that has been repeatedly exposed to brief episodes of ischemia can become tolerant to preconditioning (31, 32, 46, 47). These observations suggest that some patients with a pending myocardial infarction may have developed tolerance to protection as a result of repeated episodes of pre-infarct angina. However, we have shown that myocardium that has become tolerant to the protection by a particular stimulus (e.g., the adenosine-dependent stimulus 1IPC15) can still be protected by a preconditioning stimulus employing a different pathway (e.g., the ROS-dependent stimulus 3IPC3) or by remote preconditioning of the heart (e.g., by a brief mesenteric artery occlusion) (32). Also, pharmacological preconditioning by exogenous adenosine, which acts in part via (remote) activation of a neurogenic pathway (30), overcomes the resistance to the 1IPC15 stimulus, which employs an endogenous adenosine-dependent pathway (32, 48). It is therefore important to establish whether patients that have become tolerant to preconditioning can still benefit from protection by postconditioning during the revascularization procedure. We observed that postconditioning failed to afford protection of myocardium that had become tolerant to 1IPC15 by repeated episodes of the 1IPC15 stimulus (4IPC15). It could be argued that postconditioning might still afford some degree of protection in myocardium that has become tolerant to another preconditioning stimulus, e.g., the 3IPC3 stimulus. Unfortunately, repeated application of 3IPC3 stimuli in pilot experiments and the consequent repetitive (>12) coronary artery occlusions with the silk ligature resulted in proximal coronary artery trauma with uncertain levels of reperfusion between occlusions, hence making infarct size data difficult to interpret. As a consequence of these technical problems, we were unable to perform additional studies to test the efficacy of postconditioning in myocardium tolerant to 3IPC3. Despite these limitations, the observation in the present study that tolerance to preconditioning with 1IPC15 resulted in cross-tolerance to the cardioprotective effects of postconditioning may have important clinical implications. For example, our results suggest that patients enrolled in studies into the efficacy of postconditioning during revascularization procedures may require stratification according to the absence or presence of pre-infarct angina, as the latter may be less responsive to postconditioning therapy.

Clinical Implications. The present study shows that postconditioning does not afford additional protection after a long period of index ischemia when hearts have been preconditioned, irrespective of the signal transduction pathway employed by the preconditioning stimulus and irrespective of myocardial tolerance to preconditioning. These observations in the *in vivo* rat heart could be interpreted to suggest that future multi-center randomized trials, which are clearly needed to confirm the preliminary small clinical trials (9–12), may have to stratify patients according to the presence or absence of pre-infarct angina.

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