Endogenous angiotensin-(1-7)/Mas receptor/NO pathway mediates the cardioprotective effects of pacing postconditioning

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Abwainy A, Babiker F, Akhtar S, Benter IF. Endogenous angiotensin-(1–7)/Mas receptor/NO pathway mediates the cardioprotective effects of pacing postconditioning. Am J Physiol Heart Circ Physiol 310: H104–H112, 2016. First published October 30, 2015; doi:10.1152/ajpheart.00121.2015.—The aim of the present study was to investigate the role of the ANG-(1–7) receptor (Mas) and nitric oxide (NO) in pacing postconditioning (PPC)-mediated cardioprotection against ischemia-reperfusion injury. Cardiac contractility and hemodynamics were assessed using a modified Langendorff system, cardiac damage was assessed by measuring infarct size and creatinine kinase levels, and levels of phosphorylated and total endothelial NO synthase (eNOS) were determined by Western blot analysis. Isolated hearts were subjected to 30 min of regional ischemia, produced by fixed position ligation of the left anterior descending coronary artery, followed by 30 min of reperfusion (n = 6). Hearts were also subjected to PPC (three cycles of 30 s of left ventricular pacing alternated with 30 s of right atrial pacing) and/or treated during reperfusion with ANG-(1–7), Nω-nitro-L-arginine methyl ester, or the Mas antagonist (D-Ala7)-ANG I/II (1–7). The PPC-mediated improvement in cardiac contractility and hemodynamics, cardiac damage, and eNOS phosphorylation were significantly attenuated upon treatment with (D-Ala7)-ANG I/II (1–7) or Nω-nitro-L-arginine methyl ester. Treatment with ANG-(1–7) improved cardiac function and reduced infarct size and creatinine kinase levels; however, the effects of ANG-(1–7) were not additive with PPC. In conclusion, these data provide novel insights into the cardioprotective mechanisms of PPC in that they involve the Mas receptor and eNOS and further suggest a potential therapeutic role for ANG-(1–7) in cardiac ischemic injury.

NEW & NOTEWORTHY

Hearts can be protected from ischemic injury by pacing postconditioning. Using an isolated heart model of ischemia-reperfusion injury, we provide novel insights into the cardioprotective mechanisms of pacing postconditioning in that they involve the Mas receptor and endothelial nitric oxide synthase. The data suggest a potential therapeutic role for ANG-(1–7) in cardiac ischemic injury.

ISCHEMIC HEART DISEASE and the resulting myocardial infarction have been predicted to be the major causes of death in 2020 (24). Although reperfusion of the myocardium is essential for salvaging cardiac function, it also paradoxically leads to cardiomyocyte injury and death (17). The damaging effects of ischemia-reperfusion (I/R) injury can be limited by cardiac postconditioning, which is defined as “a series of brief mechanical interruptions of reperfusion following a specific prescribed algorithm applied at the very onset of reperfusion” (31). For example, by interrupting reperfusion after coronary artery occlusion for 1 h in a canine model (with three repeated cycles of an algorithm of 30 s of reperfusion followed by 30 s of reocclusion) and subsequent full reperfusion for 3 h, significant cardioprotection was observed. This protection was associated with an improvement in endothelial function and a reduction in tissue superoxide generation, cardiac apoptosis, microvascular injury, and infarct size (31). Improvements in left ventricular (LV) contractility and coronary vascular hemodynamics have been demonstrated by ischemic postconditioning irrespective of reperfusion time (28).

In another type of cardiac protection, rapid pacing of the heart before regional ischemia resulted in a notable protection to the heart (21). We have shown that intermittent dysynchrony, induced by ventricular pacing [pacing postconditioning (PPC)], reduced infarct size similar to that observed with ischemic postconditioning when applied at the beginning of reperfusion (4–6). PPC produced a remarkable reduction in infarct size in a clinical setting; however, an improvement in the technique used was suggested (32). Although the precise mechanisms involved in PPC are not well understood, there is evidence for differences in the upstream effectors compared with other forms of postconditioning, such as ischemic postconditioning. For example, in a previous study (4), we have shown that adenosine and ANG II, which have been proven to be important for ischemic postconditioning (18), are not involved in mediating the cardioprotection afforded by PPC (4). Thus, there is a need to characterize the underlying mechanisms involved in PPC-mediated cardioprotection after I/R injury. Furthermore, uncovering the underlying mechanisms will enhance the future use of PPC in the clinic (32).

ANG-(1–7) is a peptide member of the renin-angiotensin-aldosterone system that is cardioprotective and known to improve myocardial contractility and survival (7). We have previously reported on the cardioprotective effects of ANG-(1–7) in models of diabetes and/or hypertension (1, 6, 7). ANG-(1–7) mediates its cardioprotective effects through its G protein-coupled receptor, Mas (25), through a pathway involving activation of nitric oxide (NO) synthesis (12). ANG-(1–7) was also beneficial in protecting the ischemic heart in a preconditioning setting (15). In contrast, Zhang et al. (35) have reported a detrimental role for ANG-(1–7) in I/R injury where its blockade was deemed important for cardiomyocyte protection. Although these discrepancies were reported in preconditioning, the role of ANG-(1–7) and its receptor, Mas, in postconditioning protection against I/R injury has not been investigated.
Since ANG-(1–7) is known to be an inducer of NO in the protection against some heart diseases (9, 10), we therefore hypothesized that PPC-mediated cardioprotection involves the ANG-(1–7) receptor (Mas) and signaling via NO, a known downstream effector of ANG-(1–7) (11).

MATERIALS AND METHODS

Ethics statement. Animal treatments and handling were in accordance with international standards of animal care. The study was approved by the Ethics Committee for animal use of the Health Science Center of Kuwait University and was conducted according to the laboratory’s animal care guidelines at Kuwait University. Rats were maintained under controlled temperature (21–24°C) and humidity (50%). Animals were housed in plastic cages (2 rats/cage) and were allowed tap water and chow ad libitum. Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg) and anticoagulated with heparin (1,000 U/kg body wt) through the femoral vein. A thoracotomy was performed, and hearts were isolated while animals were completely anesthetized.

Isolated rat heart preparation. Hearts isolated from male Wistar rats weighing between 270 and 300 g (n = 9 rats/group) were used for this study. Heart cannulation and perfusion were performed as previously described (20). Briefly, the isolated heart was perfused retrogradely with freshly prepared Krebs-Hensleit buffer. The buffer was oxygenated with a mixture of O2 (95%) and CO2 (5%) at pH 7.35–7.45 and a temperature of 37.0 ± 0.5°C. Under basal control conditions, preload was kept constant at 6 mmHg. Perfusion pressure was kept constant at 50 mmHg throughout the experimental procedure in all study protocols. Perfusion pressure was measured from a branch of the aortic cannula using a Statham pressure transducer (P23 Db). Constant perfusion pressure was ensured electronically by means of the perfusion assembly (module PPCM type 671, Hugo Sachs Elektronik, Harvard Apparatus). This system permits an accurate adjustment of perfusion pressure between 5 and 150 mmHg, with an accuracy of ±1 mmHg.

Study protocol. The heart was instrumented with pacing electrodes on the right atrial (RA) appendage to keep the heart beating at the physiological heart rate of the rat and on the posterior basal LV wall during PPC. The left anterior descending coronary artery was encircled by a snare at ~0.5 cm below the atrioventricular groove, and a small rigid plastic tube was positioned between the heart and snare to ensure complete occlusion of the coronary artery. Subsequently, the heart was reperfused for 30 min. In hearts subjected to PPC, an epicardial pacing electrode was fixed to the posterior basal LV wall and connected to a pacemaker set to the required pacing frequency. Postconditioning was performed using three consecutive cycles consisting of 30 s of LV pacing alternating with 30 s of RA pacing during the first 3 min of reperfusion. Ventricular pacing done at 10% above the normal heart rate of the rat using simultaneous atrioventricular pacing (atrioventricular interval = 0 ms) to ensure complete ventricular activation initiating from the pacing electrodes.

Hearts were subdivided into eight groups (n = 9 hearts/group; Fig. 1). Control hearts were subjected only to ischemia and reperfusion without any other treatment. PPC was three episodes of 30 s of LV pacing alternated with 30 s of RA pacing. For protection with ANG-(1–7) in the control group, ANG-(1–7) (10−8 M) was administered. The effect of ANG-(1–7) on PPC protection was studied using a protocol similar to the PPC group with the addition of a Mas antagonist [(D-Ala7)-ANG II (1–7); 10−7 M]. The involvement of the Mas receptor in the protection by ANG-(1–7) was investigated using a treatment similar to the control group in the presence of ANG-(1–7) and Mas antagonist. The effects of NO synthesis on protection were studied using two groups similar to the PPC or ANG-(1–7) groups with the addition of Nω-nitro-L-arginine methyl ester (L-NAME; 100 µM). For the effects of the additivity of ANG-(1–7) and PPC, one group of animals was treated similar to the PPC protocol plus the addition of ANG-(1–7) and Mas antagonist. The effects of NO synthesis on protection by ANG-(1–7) was investigated using a treatment similar to the control group in the presence of ANG-(1–7) and Mas antagonist. The effects of NO synthesis on protection by ANG-(1–7) was investigated using a treatment similar to the control group in the presence of ANG-(1–7) and Mas antagonist. The effects of NO synthesis on protection by ANG-(1–7) was investigated using a treatment similar to the control group in the presence of ANG-(1–7) and Mas antagonist.

Protocol A: Control
Stabilization 30 min Ischemia 30 min reperfusion

Protocol B: PPC groups
Stabilization 30 min ischemia 30 min reperfusion

Protocol C: Drug administration
Stabilization 30 min ischemia 30 min reperfusion

Protocol D: PPC and the drugs in consideration
Stabilization 30 min ischemia 30 min reperfusion

Fig. 1. Schematic representation showing the experimental protocols used in the study (n = 9 rats/group). A: unprotected ischemia-reperfusion [control (Ctr)]. B: protection with pacing post-conditioning (PPC). C: perfusion of ANG-(1–7), Mas antagonist [(D-Ala7)-ANG II (1–7)], and Nω-nitro-L-arginine methyl ester (L-NAME). D: double treatment with PPC and ANG-(1–7), Mas antagonist, and L-NAME. LV, left ventricular.
LV developed pressure (DP_{\text{max}}) was derived from the online acquisition of LV systolic pressure (LVSP) by the Max-Min module (no. MMM type 668, Hugo-Sachs Electronic). This module converts the output from the DC-BA to DP_{\text{max}} by subtracting LV end-diastolic pressure (LVEDP) from the maximal LVSP. Heart function parameters were continuously computed during acclimation, ischemic, and reperfusion periods.

Cardiac damage assessment via infarct size and creatine kinase levels. Hearts were collected after 30 min of reperfusion and stored overnight at −20°C. The next day, hearts were sliced into four to five pieces from the apex to base. The slices were then incubated in 1% 2,3,5-triphenyl-2H-tetrazolium chloride solution in isotonic pH 7.4 phosphate buffer and then fixed in 4% formaldehyde. The red and pale nonstained areas of every slice were indicated manually on the image. The percentage of the infarct size of the LV was calculated for every heart. Quantification of the LV and infarct size was done using ImageJ (National Institutes of Health). Cardiomyocytes injury was evaluated by measuring creatine kinase (CK) release in the coronary effluent during the reperfusion period, as previously described by Ferrera et al. (16).

Protein extraction and Western blot analysis. At the end of the experiments, hearts were immediately frozen in liquid nitrogen and subsequently stored at −80°C for further analysis. LV tissue was homogenized in ice-cold lysis buffer, and the homogenate was centrifuged at 4,000 rpm for 10 min. The supernatant was collected, protein content was measured, and samples were aliquoted and stored for further analysis. Expression of endothelial NO synthase (eNOS) was evaluated by standard immunoblot procedures as previously described (3). Equal loading was checked by stripping and reprobing the membrane with actin antibodies. Monoclonal antibodies against total and phosphorylated eNOS (Ser^{1177}) were used (all from Cell Signaling Technology). Detection was performed with the enhanced chemiluminescence technique after incubation with a suitable secondary antibody coupled to horseradish peroxidase (Cell Signaling Technology). A computerized image-acquisition system was used for densitometric analysis.

Data analysis. Data was expressed as means ± SE. One-way ANOVA was used for repeated measures within each group and between the groups. Post hoc analysis with a Tukey test was used for further comparison. P values of <0.05 were considered statistically significant.

RESULTS

Effect of PPC on the heart. We first confirmed the beneficial effect of PPC on LV cardiac function in the perfused rat heart. Body and heart weights of the rats corrected for tibia length (74.5 ± 4.7 g/mm and 0.28 ± 0.1 mg/mm, respectively) were not significantly different between the experimental groups. Treatment of subsets of isolated hearts with the drugs used in this study separately did not show any signs of cytotoxicity (data not shown). Ischemia deteriorated heart hemodynamics (DP_{\text{max}} and LVEDP) and coronary vascular dynamics (CF and CVR). PPC significantly (P < 0.01) improved DP_{\text{max}} and normalized LVEDP compared with the respective ischemic periods (same group at the end of ischemia) and untreated control hearts (hearts subjected to ischemia with no further treatment; Fig. 2, A and B). PPC resulted in improved LV hemodynamics (Fig. 2, A and B) and coronary vascular resistance (Fig. 2, C and D).

Fig. 2. PPC protection to the heart against ischemia-reperfusion injury and the role of ANG-(1–7) in this protection (n = 9 hearts/group). Postischemic recovery in myocardial hemodynamics is shown. A: maximum developed pressure (DP_{\text{max}}). B: LV end-diastolic pressure (LVEDP). C: coronary flow (CF). D: coronary vascular resistance (CVR). Data were computed at 30 min of reperfusion and are expressed as means ± SE. *P < 0.001 compared with the respective control group; †P < 0.001 compared with the ischemic period.
in a significant ($P < 0.001$) improvement in CF compared with ischemic periods and the untreated control group (Fig. 2C). The changes that occurred in CF were associated with a considerable elevation in CVR after ischemia when no protection was applied. Protection of the hearts with PPC significantly ($P < 0.001$) normalized this elevation compared with the ischemic period and the untreated control group (Fig. 2D).

**Role of endogenous ANG-(1–7) in PPC protection.** We then assessed whether the beneficial effects of PPC on cardiac function were mediated via the ANG-(1–7)/Mas receptor pathway. The role of endogenous ANG-(1–7) in PPC cardioprotection was studied using (D-Ala7)-ANG I/II (1–7), a Mas receptor blocker that completely abrogated PPC protection to the heart. Administration of the Mas receptor antagonist postischemically attenuated the DPmax improvement shown by PPC compared with the ischemic period and the untreated control group (Fig. 2A). The presence of Mas antagonist reversed the improvement of LVEDP observed during PPC and elevated it to a value comparable with that of the ischemic period and the untreated control group (Fig. 2B). The improvement of CF and CVR shown by PPC was completely abrogated by Mas antagonist perfusion compared with the ischemic period and the untreated control group (Fig. 2, C and D).

**Effect of administration of exogenous ANG-(1–7) on heart function.** We next evaluated whether postischemic administration of ANG-(1–7) could mimic the beneficial effects of PPC on cardiac function. Hearts perfused with exogenous ANG-(1–7) administered postischemically showed a significant ($P < 0.01$) improvement in DPmax compared with the ischemic period and the untreated control group (Fig. 2A). The elevation of LVEDP caused by ischemia was significantly ($P < 0.01$) normalized by exogenous ANG-(1–7) treatment compared with the ischemic period and the untreated control group (Fig. 2B). Ischemia was associated with a considerable decrease in CF and elevation in CVR levels. ANG-(1–7) treatment significantly ($P < 0.001$) normalized CF and CVR levels compared with the respective ischemic period and the untreated control group (Fig. 2, C and D). Interestingly, all these improvements in heart hemodynamics and coronary vascular dynamics were abrogated by the Mas antagonist (Fig. 2, A–D).

**Role of NO in PPC cardioprotection.** To study the role of NO as a downstream effector of PPC and/or ANG-(1–7)-mediated cardioprotection, we used L-NAME as a blocker of NO synthesis. L-NAME completely abrogated the protection afforded by PPC. The improvement of DPmax shown by PPC alone was attenuated by the presence of L-NAME to a level significantly ($P > 0.05$) below that of the ischemic period and the untreated control group (Fig. 3A). The increase in LVEDP caused by ischemia was further enhanced in the presence of L-NAME to reach a value significantly ($P < 0.05$) worse than that of the ischemic period and untreated control hearts (Fig. 3B). L-NAME blocked the improvement of CF caused by PPC, reaching a value significantly ($P < 0.05$) below that of the ischemic period and the untreated control group (Fig. 3C).

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**Fig. 3.** PPC protection to the heart against ischemia-reperfusion injury and the role of ANG-(1–7) and nitric oxide (NO) in this protection (n = 9 hearts/group). Postischemic recovery in myocardial hemodynamics is shown. A: DPmax. B: LVEDP. C: CF. D: CVR. Data were computed at 30 min of reperfusion and are expressed as means ± SE. *$P < 0.001$ compared with the respective control group; †$P < 0.001$ compared with the ischemic period; ‡$P < 0.05$ compared with the ischemic period.
CVR did not show any improvement in the presence of L-NAME but was significantly ($P < 0.05$) worse than the ischemic period and untreated control levels (Fig. 3D).

The involvement of NO in mediating cardioprotection induced by exogenous ANG-(1–7) was studied using L-NAME, which completely abrogated the protection afforded by ANG-(1–7). The improvement of $D_{P\text{max}}$ shown by ANG-(1–7) alone was attenuated by the presence of L-NAME to a level that was almost similar to that of the ischemic period and the untreated control group (Fig. 3A). The elevation of LVEDP caused by ischemia was further elevated in the presence of L-NAME compared with the ischemic period and untreated control hearts (Fig. 3B). L-NAME attenuated the improvement of CF caused by PPC and lowered it to a level similar to that of the ischemic period and the untreated control group (Fig. 3C). CVR did not show any improvement in the presence of L-NAME but was worse than that in the ischemic period and the untreated control group (Fig. 3D).

These findings were further confirmed by immunochemistry showing the expression levels and phosphorylation of eNOS in various treatments. The expression and phosphorylation of eNOS were significantly ($P < 0.01$) increased by PPC in paced hearts compared with untreated control hearts (Fig. 4). This increase was completely abrogated by the blockade of PPC protection by L-NAME. Interestingly, a significant ($P > 0.001$) increase in eNOS expression levels and phosphorylations was observed when the other protective molecules, such as ANG-(1–7), were used separately in the presence of L-NAME or when they were combined with PPC compared with the untreated control group ($P < 0.001$). Treatment of the heart with the Mas antagonist completely blocked the protective effect of PPC and decreased NO expression levels and phosphorylation (Fig. 4).

Protective effects of combined treatments against I/R injury. To investigate any potential synergy in cardioprotection, the effect of combining ANG-(1–7) administration together with PPC on cardiac function was studied. The combination of exogenous ANG-(1–7) and PPC resulted in a significant improvement ($P < 0.001$) in $D_{P\text{max}}$ compared with the ischemic period and the untreated control group (Fig. 5A). The elevation of LVEDP caused by the ischemic injury was significantly ($P < 0.001$) reduced by the combination of ANG-(1–7) and PPC compared with the ischemic period and the untreated control group (Fig. 5B). CF was significantly ($P < 0.01$) improved by this combination compared with the ischemic period and the untreated control group (Fig. 5C). CVR was significantly ($P < 0.001$) normalized compared with the ischemic period and the untreated control group (Fig. 5D). However, this improvement was not significantly different from that of these regimens when they were used separately (Fig. 5, A–D).

Cardiac injury evaluation. We next assessed whether the beneficial effects of PPC and various treatments on cardiac function could be correlated with a reduction in cardiac damage by measuring infarct size (Fig. 6A) and CK levels (Fig. 6B). Ischemia and reperfusion resulted in a large infarcted area and high CK level in control hearts and other hearts that were not subjected to any of the protective measures (Fig. 6). Both PPC or treatment with ANG-(1–7) resulted in a significant ($P < 0.001$) decrease in the infarct size and CK levels compared with the untreated control group. The reduction in infarct size and CK levels with PPC or

Fig. 4. Western blot showing the phosphorylation and expression of endothelial NO synthase (eNOS). A: Western blot showing the expression of total (T-)-eNOS and phosphorylated (P-)-eNOS corrected to actin. B: P-eNOS corrected to actin. C: expression levels of T-eNOS corrected to actin. Values are means ± SE for 3 individual experiments. *$P < 0.001$ compared with the control group. **$P < 0.001$ compared with PPC.
treatment with ANG-(1–7) was completely reversed by Mas antagonist or l-NAME treatments (Fig. 6). The combination of PPC and ANG-(1–7) resulted in a significant reduction in the infarct size and CK levels similar to that attained by PPC or ANG-(1–7) alone (Fig. 6A).

**DISCUSSION**

PPC is known to produce pronounced cardiac protection after I/R injury (4, 30), but the underlying mechanisms of this protection are not fully understood. The present study demonstrated, for the first time, that the cardioprotective effects of PPC are mediated by the endogenous ANG-(1–7)/Mas receptor axis and increased synthesis and phosphorylation of eNOS. Furthermore, this study showed that postischemic administration of exogenous ANG-(1–7) is also cardioprotective to a level that is similar to that obtained by PPC or ANG-(1–7) alone (Fig. 6A).

The cardioprotective effects of PPC are mediated via endogenous ANG-(1–7)/Mas receptor. This study showed that the cardioprotective effects of PPC could be abrogated by administration of a selective Mas receptor blocker, implying that the endogenous ANG-(1–7)/Mas receptor axis is important in the survival of cardiomyocytes after I/R injury and that its activation is integral to the cardioprotection attained by PPC. The fact that exogenous ANG-(1–7) administered postischemically also generated cardioprotection against I/R injury at a level similar to that attained by PPC provided further support for this assertion (see Fig. 2). The fact that exogenous ANG-(1–7) did not exert additional cardioprotection over and above that of PPC alone implies that PPC activation of ANG-(1–7)/Mas receptor is optimal. Importantly, our study shows that postischemic administration of ANG-(1–7) afforded significant cardiac protection to a level similar to that offered by PPC and thus may serve as an alternative treatment option after cardiac ischemic events (Fig. 7). Our findings are supported by other recent studies where exogenous ANG-(1–7) administered before ischemic injury was also cardioprotective in diabetes- and/or hypertension-induced cardiac dysfunction (1, 6, 7). The presence of ANG-(1–7) in the myocardium (13) and the local production of ANG-(1–7) by cardiomyocytes (27) suggest a definite role for this peptide in the heart. Its beneficial role was highlighted by the protection triggered by ANG-(1–7) against I/R damage when applied before the onset of ischemia (preconditioning) (1). This cardioprotection may be through a pathway opposing the effect of ANG II or directly by producing coronary vasodilation (13) but appears to be mediated via its receptor, Mas (2), and/or possibly via an indirect way involving ANG II type 1 and/or type 2 receptors (8). Interestingly, many receptors have been shown to be involved in postconditioning protection to the heart (33). Specifically, bradykinin receptors have been proven to be involved in pacing...
preconditioning to the heart (21). Both ANG-(1–7) and bradykinin play their protective role through a pathway involving NO release (23). The involvement of ANG-(1–7) and bradykinin in pacing protection to the heart and the presence of bradykinin receptors downstream to ANG-(1–7) (19) may suggest receptor cross talk between them; however, this still needs to be validated by further research. The data from the present study suggest that the cardioprotective effects of endogenous ANG-(1–7) are also mediated via its Mas receptor, although the role of other ANG receptors was not examined and cannot be excluded.

The cardioprotective effects of PPC and ANG-(1–7) are mediated via NO. NO is known to be involved in cardioprotection, but its role in PPC is not completely understood. Importantly, the fact that blockade of NO synthesis by L-NAME abrogated the beneficial effects of PPC supports the notion that the cardioprotective effects of PPC are, at least partly, mediated via activation of NO. In this study, PPC and ANG-(1–7) significantly increased the expression levels of NO. L-NAME and Mas antagonist completely reversed the protective effect of PPC and that of exogenous ANG-(1–7) and the increase in eNOS expression levels. These results
imply a downstream role of NO in mediating cardioprotection in both strategies and lend further support to our finding that PPC-mediated cardioprotection occurs via a ANG-(1–7)/Mas receptor/NO pathway. It should, however, be noted that treatment with the Mas receptor antagonist completely abrogated the cardioprotection afforded by PPC, whereas, surprisingly, L-NAME resulted in a deterioration in cardiac function below that of ischemia alone. This could likely represent blockade of NO synthesis by L-NAME beyond that released by the Mas receptor alone and may affect the release of NO in response to stimulation of other G protein-coupled receptors, such as bradykinin receptors, which are known to play a role in PPC-induced cardioprotection (10). However, this explanation is not fully supported by our data that suggest that both treatments had similar inhibitory effects on eNOS (Fig. 4), implying that L-NAME may have other unexplained effects in the heart that lead to a deterioration of cardiac function beyond that observed with Mas receptor antagonism, a subject that clearly requires further investigation.

Our data are also supported by similar findings in the coronary vasculature (26) where ANG-(1–7)-mediated protection occurred via a pathway involving NO synthesis. However, administration of NO during reperfusion reportedly reduces the infarct size and posts ischemic coronary vascular endothelial injury (22), indicating the usefulness of NO for this protection. Our finding that blockade of NO synthesis before the onset of reperfusion reversed the infarct-sparing effect of postconditioning is similar to that of Yang et al. (34). Indeed, a direct vasodilatation role for ANG-(1–7) has also been reported (13), and a vasodilatation role has also been reported for a Mas receptor/NO pathway (2). ANG-(1–7) is also known to improve cardiomyocyte contractility in isolated heart rat preparations (14). Based on the involvement of NO in ANG-(1–7) protection, one could speculate that ANG-(1–7) uses a pathway similar to that of classical postconditioning as it has been suggested that NO synthesis protects by inhibiting the opening of the mitochondrial permeability transition pore, which is an effector targeted by postconditioning (29). This finding in itself may explain the lack of additive effects when ANG-(1–7) is combined with PPC.

A potential limitation of this study is that the ex vivo isolated perfused heart model used does not allow an accurate assessment of the individual area of risk in normalizing infarct size due to the presence of the balloon and the retrograde perfusion of the heart. To mitigate variations in the area of risk, we standardized the occlusion point in all hearts and the infarct size data were normalized to the LV area. The fact that infarct size data could be corroborated with data on CK levels, another measure of cardiac injury, further mitigated this limitation. Since inflammatory cells such as leukocytes play an important role in I/R injury via a wide range of mechanisms, including TNF-α signaling, another potential limitation of our ex vivo isolated heart model is that it will not take into account the role of leukocytes compared with the situation in an in vivo working heart model.

Conclusions. The present study has identified a novel involvement of endogenous ANG-(1–7)/Mas receptor via its downstream effector (eNOS) in mediating the cardioprotection afforded by PPC. Our data further suggests that the exogenous administration of ANG-(1–7) postischemically might represent a novel pharmacological approach for the treatment of myocardial I/R injury as it induces cardioprotection similar to that of PPC. The latter finding may have important clinical implications as there is a great need to identify effective pharmacological agents that can be safely administered postischemically, e.g., after a heart attack. Thus, our data indicate that further preclinical and clinical studies are needed to fully evaluate the potential activation of the angiotensin-converting enzyme 2/ANG-(1–7)/Mas receptor as a novel pharmacological intervention strategy for postischemic treatment of heart conditions such as myocardial infarction.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

MECHANISMS OF PACING POSTCONDITIONING PROTECTION


