Preconditioning actions of aldosterone through p38 signaling modulation in isolated rat hearts

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Abstract

Although persistent excessive actions of aldosterone have unfavorable effects on the cardiovascular system, primarily via mineralocorticoid receptor (MR)-dependent pathways, the pathophysiological significance of aldosterone cascade activation in heart diseases has not yet been fully clarified. We herein examined the effects of short-term aldosterone stimulation at a physiological dose on cardiac function during ischemia–reperfusion injury (IRI). In order to study the effects of aldosterone preconditioning, male Wistar rat Langendorff hearts were perfused with 10^{-9} mol/l of aldosterone for 10 min before ischemia, and the response to IRI was assessed. Although aldosterone did not affect the baseline hemodynamic parameters, preconditioning actions of aldosterone significantly improved the recovery in left ventricular contractility and left ventricular end-diastolic pressure associated with a reduced activity of creatine phosphokinase released into the perfusate after ischemia–reperfusion. Notably, the MR inhibitor eplerenone did not abrogate these beneficial effects. Biochemical analyses revealed that p38MAPK phosphorylation was significantly increased during aldosterone preconditioning before ischemia, whereas its phosphorylation was substantially attenuated during sustained ischemia–reperfusion, compared with the results for in the non-preconditioned control hearts. This dual regulation of p38MAPK was not affected by eplerenone. The phosphorylation levels of other MAPKs were not altered by aldosterone preconditioning. In conclusion, the temporal induction of the aldosterone cascade, at a physiological dose, has favorable effects on cardiac functional recovery and injury following ischemia–reperfusion in a MR-independent manner. Phasic dynamism of p38MAPK activation may play a key role in the physiological compensatory pathway of aldosterone under severe cardiac pathological conditions.

Key Words

- ▶ aldosterone
- ▶ preconditioning
- ▶ ischemia–reperfusion injury
- ▶ p38MAPK

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Introduction

Aldosterone plays a pivotal role in the pathophysiology of cardiovascular diseases. There is increasing evidence of unfavorable effects of aldosterone on the cardiovascular system, in experimental models as well as large

clinical trials (Pitt *et al.* 2003), primarily via mineralocorticoid receptor (MR)-dependent actions (Caprio *et al.* 2008, Matsui *et al.* 2008, Rickard *et al.* 2012, Shen & Young 2012, Sherajee *et al.* 2012, Diaz *et al.* 2014, Nolly *et al.* 2014).

However, the pathophysiological significance of the increase (even by a small amount) in aldosterone levels both in circulation and local tissue, as well as its cascade activation in severe heart diseases (Mizuno *et al.* 2001, Nakamura *et al.* 2004), such as myocardial infarction (Beygui *et al.* 2006), has not yet been fully clarified.

Preconditioning induced by either brief periods of ischemia or short-term stimulation of neurohumoral factors, such as catecholamines and erythropoietin before ischemia–reperfusion, results in cardioprotective effects against ischemia–reperfusion injury (IRI; Marais *et al.* 2001, 2005, Sanada *et al.* 2001, Cai & Semenza 2004, Turrell *et al.* 2011). The signaling pathways involved in preconditioning have been investigated intensively, and PI3K–Akt–GSK3 as well as the MAPK family (ERK, c-Jun N-terminal kinases (JNK) and p38) has been shown to play a part in major cascades (Mocanu *et al.* 2000, Tong *et al.* 2000, Marais *et al.* 2001, 2005, Sanada *et al.* 2001, Cai & Semenza 2004, Hausenloy & Yellon 2006, Schmidt *et al.* 2010, Turrell *et al.* 2011).

Aldosterone, one of the major neurohumoral factors acting under critical cardiac conditions (such as acute-phase ischemic heart disease), exerts biphasic effects on somatic cells, including those in the myocardium (Connell & Davies 2005). While the prolonged pathological actions of excessive aldosterone are largely mediated through the activation of an MR-dependent pathway, there is a rapid, MR-independent action representing a physiological compensatory mechanism that may function to improve cardiac output during heart failure. In fact, previous studies have demonstrated that the rapid actions of aldosterone exert positive inotropic effects in the heart (Barbato *et al.* 2002, 2004, Chai *et al.* 2005, 2006, 2010). Moreover, we recently reported that aldosterone has favorable effects on cardiomyocytes during the early phase in an *in vitro* model of neonatal rat cardiomyocytes (NRCMs), partly due to the acute activation of a MR-independent cascade, whereas the persistent activity of aldosterone produces pathological effects in a MR-dependent manner (Yamamuro *et al.* 2006, Nagoshi *et al.* 2012). Therefore, we herein propose a hypothesis that the rapid MR-independent actions of aldosterone at a physiological dose initially provide cardioprotective effects under critical pathological conditions as a possible compensatory mechanism. In order to test the functional significance of the temporal induction of the aldosterone cascade, we studied the effects of short-term aldosterone stimulation before IRI (namely, ‘aldosterone preconditioning’) and examined the response in the hearts in an *ex vivo* model of IRI.

Materials and methods

Experiments in Langendorff hearts

All animal procedures conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Research Committee at the Jikei University School of Medicine. Male Wistar rats (weight: 250–300 g) were heparinized (1000 IU/kg, i.p.) and anesthetized (pentobarbital, 60 mg/kg, i.p.). The hearts were rapidly excised and the aortae were cannulated onto a Langendorff apparatus, followed by retrograde-perfusion at a constant pressure (80 mmHg) with modified Krebs–Henseleit buffer, as previously described (Nagoshi *et al.* 2005). A water-filled balloon catheter was introduced into left ventricles to record the hemodynamic parameters.

Ischemia–reperfusion model

The experimental protocols are shown in Fig. 1. After a stabilization period of 15 min, the hearts were perfused with the control samples for another 10 min before ischemia–reperfusion in order to measure the baseline pre-ischemia cardiac function. In the aldosterone preconditioning groups, 10^{-9} mol/l of aldosterone (Wako Pure Chemical Industries, Osaka, Japan) with or without 10^{-6} mol/l of the MR antagonist eplerenone (Sigma–Aldrich, the dose used in the previous study using a rat Langendorff perfusion model in order to substantially block the MR-dependent action (Chai *et al.* 2006), as well as on the basis of the pharmacokinetics of eplerenone after oral administration to humans (Cook *et al.* 2003)), 10^{-7} mol/l of the glucocorticoid receptor (GR)/progesterone receptor antagonist RU486 (Sigma–Aldrich), 10^{-7} mol/l of the G protein-coupled estrogen receptor (GPER, previously known as G protein-coupled receptor 30 (GPR30)) antagonist G15 (Cayman Chemical, Ann Arbor, MI, USA), or 10^{-5} mol/l of p38MAPK inhibitor SB203580 (Tocris, Bristol, UK) was added to the buffer during the 10-min pre-ischemia perfusion period. Where indicated, 10^{-6} mol/l of eplerenone alone was also perfused during the 10-min pre-ischemia period only. Water-insoluble reagents, such as eplerenone, RU486, and G15, were dissolved in dimethylsulfoxide (DMSO), and the solvent concentrations were identically maintained in the control groups. The preliminary experiments showed that the presence of equivalent amounts of DMSO in the perfusate did not modify cardiac performance. Subsequently, global ischemia was applied by eliminating flow for 30 min

Figure 1

Experimental protocol showing the duration and time course of ischemia/reperfusion. The hearts were frozen at the points in the protocol indicated by solid arrows. Creatine phosphokinase (CPK) activity released

into the perfusate was measured at the points in the protocol indicated by dashed arrows. Aldo, aldosterone; Epl, eplerenone.

followed by 40 min of reperfusion. In the signaling analysis, the individual perfused hearts were snap-frozen in liquid nitrogen at the indicated time points and stored at -80°C before protein extraction (Fig. 1).

Cardiac enzyme measurement

Creatine phosphokinase (CPK) levels were measured in the effluent at the time points indicated in the experimental protocols (Fig. 1) using an enzymatic activity assay.

Immunoblotting

Immunoblotting was carried out as described previously (Nagoshi *et al.* 2005, 2012) using primary antibodies to phospho-Akt (Ser473), phospho-GSK3 β (Ser9), Akt (Cell Signaling Technology, Danvers, MA, USA), GSK3 β , phospho-p38 (Thr180/Tyr182), p38, phospho-ERK (Thr202/Tyr204), ERK, phospho-JNK (Thr183/Tyr185) or JNK (BD Transduction Laboratories, Franklin Lakes, NJ, USA). The signals were detected using chemiluminescence, and the band intensity was quantified using the Multi Gauge software program (Version 3.1; Fujifilm, Tokyo, Japan).

Statistical analysis

The data are presented as the mean $\pm$ S.E.M. of at least four independent experiments. The hemodynamic parameters and CPK levels were compared between the groups using a one-way ANOVA followed by *post-hoc* Bonferroni

and Tukey's tests for multiple comparison correction. The phosphorylation levels of intracellular kinase signaling were compared using the Wilcoxon rank-sum test. A value of $P < 0.05$ was considered to be significant.

Results

Effects of aldosterone preconditioning on functional recovery and cardiac injury after IRI

The baseline cardiac function measured at the end of 10 min of pre-ischemia perfusion was not significantly affected by aldosterone preconditioning with or without eplerenone (Table 1). After 30-min global ischemia followed by 40-min reperfusion, aldosterone preconditioning significantly improved the left ventricular developed pressure (LVDP) recovery compared with that observed in the nontreated control hearts (70.5 ± 3.1 vs $55.3 \pm 3.0\%$ recovery of baseline, $P < 0.05$, Fig. 2A and B). Left ventricular end-diastolic pressure (LVEDP) during reperfusion was also significantly lower in the aldosterone-preconditioned hearts compared with that observed in the control hearts (29.2 ± 3.7 vs 44.9 ± 2.6 mmHg at the end of IRI, $P < 0.03$, Fig. 2C). Notably the administration of eplerenone, a selective MR antagonist, during aldosterone preconditioning did not abrogate these beneficial effects (Fig. 2A, B and C). The administration of eplerenone alone during the pre-ischemic period only did not significantly affect the recovery of the cardiac function after IRI (Supplementary Figure 1A, see section on supplementary data given at the end of this article). In the context of an

Table 1 Data on baseline cardiac function of the ex vivo perfused hearts

	Control (n=23)	Aldo (n=20)	Aldo + Epl (n=9)
LVSP (mmHg)	150.0 ± 3.3	146.5 ± 3.7	156.9 ± 4.3
LVEDP (mmHg)	7.4 ± 0.4	6.4 ± 0.4	8.4 ± 0.5
LVDP (mmHg)	142.6 ± 3.4	140.1 ± 3.9	148.5 ± 4.2
LV + dp/dt (mmHg/s)	4985.2 ± 137.4	4757.3 ± 155.9	4941.0 ± 135.0
LV - dp/dt (mmHg/s)	-2839.9 ± 75.3	-2751.0 ± 75.0	-2955.2 ± 78.8
Heart rate (bpm)	229.8 ± 4.5	233.9 ± 4.4	226.2 ± 4.1
Coronary flow (ml/min)	11.7 ± 0.3	11.7 ± 0.4	11.5 ± 0.3

LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LVDP, left ventricular developed pressure.

MR-independent cascade, the effects of aldosterone in improving the cardiac functional recovery may be mediated via GR-dependent (Rossier *et al.* 2008) or GPER-dependent (Krug *et al.* 2011) actions. However, neither RU486 (GR inhibitor) nor G15 (GPER inhibitor) affected the cardiac function at baseline or in terms of LVDP recovery after IRI (Fig. 2D), indicating that the functional effects of aldosterone preconditioning observed

in the current study may be mediated via MR-, GR- or GPER-independent actions.

The activity of CPK released into the perfusate during reperfusion was measured as an index of myocardial injury. During baseline pre-ischemia perfusion, no CPK activity was detectable. After IRI, the CPK activity was significantly reduced in the aldosterone-preconditioned hearts compared with that observed in the control hearts

Figure 2

Cardiac functional recovery after ischemia-reperfusion injury. LVDP profiles during ischemia-reperfusion (A), LVDP recovery (percentage of baseline value) measured at the indicated time points (B), and LVEDP profiles during ischemia-reperfusion (C) in the control hearts (white squares and bars; $n=23$), aldosterone-preconditioned hearts (Aldo, black squares and bars; $n=20$) and aldosterone-preconditioned hearts treated with eplerenone

infusion (Aldo + Epl, gray squares and bars; $n=9$). (D) LVDP recovery (percentage of baseline value) measured at the end of the 40-min reperfusion period in the aldosterone-preconditioned hearts treated with or without either RU486 or G15 (Aldo + RU486 or G15; $n=7$ each). * $P<0.05$, control vs Aldo; † $P<0.05$ control vs Aldo + Epl; ‡ $P<0.05$ control vs Aldo + RU486; # $P<0.05$ control vs Aldo + G15.

Figure 3

(A) CPK profiles in the effluent collected during the reperfusion period. (B) Area under the curve (AUC) was calculated from the CPK profile shown in (A) ($n \geq 7$ for each group). * $P < 0.05$ control vs Aldo; † $P < 0.05$ control vs Aldo + Epl.

at 10 min of reperfusion (16.9 ± 3.1 vs 48.6 ± 13.7 U/l, $P < 0.02$, Fig. 3A). Moreover, the total amount of CPK released during the entire reperfusion period, as indicated by the area under the curve (AUC) for the CPK level, was significantly decreased by aldosterone preconditioning (84.5 ± 16.6 vs 146.0 ± 24.8 , $P < 0.05$, Fig. 3B). Again, the administration of eplerenone did not influence the reduction in CPK achieved by aldosterone preconditioning (Fig. 3A and B).

Together, these data indicate that aldosterone preconditioning improves cardiac functional recovery and reduces myocardial injury after IRI in a MR-independent manner.

Effects of aldosterone preconditioning on intracellular kinase signaling activation

In order to explore possible mechanisms underlying these results, we next examined signaling regulated by aldosterone preconditioning. It has been reported that the acute activation of Akt signaling during IRI induced by ischemic preconditioning or other factors is cardioprotective (Tong *et al.* 2000, Cai & Semenza 2004, Nagoshi *et al.* 2005, Hausenloy & Yellon 2006). Moreover, we recently demonstrated that aldosterone has favorable effects on cardiomyocytes due to the acute activation of a MR-independent cascade through PI3K–Akt *in vitro* (Nagoshi *et al.* 2012). Therefore, we first examined Akt signaling and found that aldosterone perfusion for 10 min significantly increased phosphorylation of Akt and that this was inhibited by eplerenone, while no significant change was observed in phosphorylation of GSK3 β , one of the downstream effectors of Akt (Fig. 4). Aldosterone preconditioning with or without eplerenone did not exert any significant effects on Akt–GSK3 β phosphorylation during either the early or late phase of reperfusion.

MAPKs also play critical roles in cardioprotective effects during IRI (Mocanu *et al.* 2000, Marais *et al.* 2001, 2005, Sanada *et al.* 2001, Hausenloy & Yellon 2006, Schmidt *et al.* 2010, Turrell *et al.* 2011). Among the three major MAPK signaling pathways, we found that p38MAPK activation is dramatically altered by aldosterone preconditioning during IRI (Fig. 5). Phosphorylation of p38 was significantly increased by aldosterone at the end of the 10-min baseline period of pre-ischemia perfusion. In contrast, phosphorylated p38 levels were markedly reduced in the aldosterone-preconditioned hearts compared with those observed in the nontreated controls during both the early and late phases of reperfusion. This rapid dual p38 regulation was not affected by eplerenone, which was consistent with the functional data. The administration of eplerenone alone during the pre-ischemic period only did not change phosphorylated p38 level significantly (Supplementary Figure 1B). The phosphorylation of ERK and JNK was not significantly altered by aldosterone preconditioning.

In order to better understand the significance of the rapid pre-ischemic induction of p38MAPK signaling induced by aldosterone preconditioning, we examined the effects of SB203580 (a potent p38 inhibitor) perfusion during aldosterone preconditioning on cardiac functional recovery after IRI. Perfusion with 10^{-6} mol/l of SB203580 alone or SB203580 + aldosterone during 10-min pre-ischemic period caused a rapid positive inotropic effect,

Figure 4

Phosphorylation of Akt and GSK3 β was evaluated in hearts treated with or without ischemia–reperfusion for the indicated periods shown in Fig. 1. Representative immunoblots obtained using the indicated antibodies are

shown. Averaged densitometry data normalized to the control at the same time points are shown in the bar graphs ($n=3$ –4 for each group). * $P<0.05$, control vs Aldo; † $P<0.05$ Aldo vs Aldo + Epl.

although there were no synergistic effects (138.8% increase in LVDP). After IRI, the recovery achieved with aldosterone preconditioning was blunted by SB203580 to a level similar to that of the control (Fig. 6), indicating that transient p38MAPK activation during aldosterone preconditioning plays a key role in cardioprotection.

Discussion

In the present study, we found that the temporal induction of the aldosterone cascade, at a physiological dose, results in increased cardiac functional recovery and decreased myocardial injury after ischemia–reperfusion in a MR-independent manner. Our results indicate a critical role of the phasic dynamism of p38MAPK activation in aldosterone preconditioning of the heart during IRI. A transient increase in p38 phosphorylation was observed during the pre-ischemic period, while attenuation of its phosphorylation was observed during ischemia–reperfusion; these effects may be deeply involved in the cardioprotective effects of aldosterone preconditioning.

It has been well documented that the persistent excessive actions of aldosterone, commonly by a combination of salt loading, cause adverse cardiovascular

effects primarily via MR-dependent pathways (Matsui *et al.* 2008, Shen & Young 2012). On the other hand, ourselves and others have recently reported that aldosterone exerts transient but favorable effects on cardiocytes *in vitro*, mainly via rapid MR-independent actions (Yamamuro *et al.* 2006, Bunda *et al.* 2009, Nagoshi *et al.* 2012). In agreement with these findings, the present *ex vivo* study demonstrated the rapid, favorable effects of aldosterone on cardiac functional recovery and injury during ischemia–reperfusion, although aldosterone did not affect the baseline cardiac function (Table 1), congruent with the findings of previous reports by other groups (Fujita *et al.* 2005, Matsui *et al.* 2007). However, our results do not completely agree with the findings of several other studies showing that rapid, MR-independent actions of aldosterone induce positive inotropic effects at baseline (Barbato *et al.* 2002, 2004, Chai *et al.* 2005, 2006, 2010), while the administration of aldosterone results in no significant effects (Chai *et al.* 2005, Schmidt *et al.* 2010), but rather results in detrimental effects (Fujita *et al.* 2005, Mihailidou *et al.* 2009) on cardiac functional recovery and injury after ischemia–reperfusion. This discrepancy may be explained by the differences in the experimental conditions and the time course of aldosterone stimulation: temporal induction of the aldosterone cascade, at a physiological dose

Figure 5

Phosphorylation of MAPKs signaling pathways was evaluated in hearts treated with or without ischemia-reperfusion for the indicated periods shown in Fig. 1. Representative immunoblots obtained using the indicated

antibodies are shown. Averaged densitometry results normalized to the control at the same time points are shown in the bar graphs ($n=4$ for each group). * $P<0.05$, control vs Aldo; † $P<0.05$ control vs Aldo + Epl.

(a relatively lower dose than that commonly used in previous studies), during the pre-ischemic period only (not during the entire IRI protocol) is crucial for the ability of aldosterone to exert favorable effects on the heart during IRI.

Mihailidou and colleagues previously demonstrated that spironolactone protects the heart from IRI, not merely by excluding aldosterone and/or glucocorticoids from MR but also via its inverse agonist activity against MR (Mihailidou *et al.* 2009). It is also possible that eplerenone acts as a partial agonist under ligand-free conditions, such as *ex vivo* isolated heart models. In this study, however, neither LVDP recovery nor p38MAPK signaling was affected by the administration of eplerenone alone during the 10-min pre-ischemic period only (Supplementary Figure 1A). The MR antagonists displayed cardioprotective effects when administered under pathological conditions, such as IRI, not at baseline, whereas the purpose of applying eplerenone in this study was to block

MR during aldosterone preconditioning only. Therefore, the recovery of cardiac function as well as signaling activation, including that involving p38MAPK to MR antagonists, may differ when eplerenone is administered for 10 min before ischemia in addition to the entire 40-min reperfusion period. Moreover, the cardioprotective effects of MR antagonists may be more salient *in vivo*, where other MR ligands (e.g. glucocorticoids) and miscellaneous endogenous factors that modulate MR (e.g. salt loading, high glucose) exist. Taken together, the lack of effects of eplerenone in the current *ex vivo* study does not exclude the well-documented cardioprotective effects of MR antagonists demonstrated in both experimental and clinical studies.

The preconditioning effects of aldosterone may also involve GR or GPER. However, neither GR nor GPER inhibitors affected the cardiac function, at least in the current protocol used to investigate the pre-ischemic

Figure 6

Effects of the p38MAPK inhibitor (SB203580) on cardiac functional recovery after ischemia–reperfusion injury. LVDP recovery (percentage of baseline value) measured at the end of the 40-min reperfusion period in aldosterone-preconditioned hearts treated with or without SB203580 is shown (Aldo + SB203580; $n = 7$). * $P < 0.05$ control vs Aldo; ‡ $P < 0.05$ Aldo vs Aldo + SB203580.

rapid actions of aldosterone at its physiological dose (Fig. 2D). It has been reported that GR is activated by aldosterone in cardiomyocytes, but at a relatively high dose (over 10^{-8} M) for long-term stimulation, compared with that observed in the current study (Rossier *et al.* 2008). It is more likely that rapid MR-independent actions are mediated via GPER signaling (Deschamps & Murphy 2009, Krug *et al.* 2011), although the aldosterone–GPER pathway may be activated in a cell-type-specific manner (vascular cells rather than cardiomyocytes) (Gros *et al.* 2011, 2013). Moreover, this pathway dramatically activates ERK rather than p38MAPK, thus indicating that aldosterone–GPER signaling is not the major cascade, at least in the present model.

We recently reported that short-term aldosterone stimulation has favorable effects on cardiomyocytes due to the acute activation of a MR-independent cascade via PI3K–Akt signaling in an *in vitro* NRCMs model (Nagoshi *et al.* 2012). Consistent with this finding, there was a significant increase in phosphorylation of Akt during aldosterone preconditioning in the current study, although the increased level of GSK3 β observed at the same time point was variable (Fig. 4). Moreover, this increased Akt phosphorylation was inhibited by eplerenone, consistent with the results recently reported by

others (Nolly *et al.* 2014). Furthermore, there were no significant differences in Akt–GSK3 phosphorylation levels between the groups during the reperfusion period. A variety of important differences between the *in vitro* NRCMs and *ex vivo* adult whole-heart tissues (containing cardiomyocytes as well as cardiac fibroblasts, endothelial cells, vascular smooth muscle cells, and tissue-resident macrophages) may have contributed to the differences in observations. In addition, it has been recently reported that the cardioprotective effects of aldosterone achieved via Akt signaling activation are observed exclusively under high-glucose conditions (Fazal *et al.* 2014), consistent with results from our previous study (Nagoshi *et al.* 2012). Taken together, even if Akt signaling is partially involved in the cardioprotective effects of aldosterone preconditioning, its contribution was minor in the current study.

The cardioprotective effects of aldosterone preconditioning observed in this study were associated with the phasic dynamism of p38MAPK phosphorylation in a MR-independent manner (Fig. 5). A similar phasic pattern of p38 phosphorylation (activation) has been reported by other groups in miscellaneous cardiac preconditioning models induced by short-term repetitive ischemia as well as catecholamines associated with improvements in cardiac functional recovery and reduced cardiac injury (Marais *et al.* 2001, 2005, Sanada *et al.* 2001). p38 exerts both favorable and unfavorable effects on the heart, which probably depend on the intensity and duration of p38 activation (Mocanu *et al.* 2000). Previous studies using other systems of cardiovascular cells have demonstrated that p38 can be phosphorylated and activated by aldosterone via both MR-dependent and rapid MR-independent pathways, consistent with the findings from this study (Callera *et al.* 2005a,b, Lopez-Andres *et al.* 2008). It has been reported that the short-term induction of p38 phosphorylation in cardiomyocytes contributes to the activation of sarcolemmal K_{ATP} channels, which is a major common mechanism responsible for preconditioning (Turrell *et al.* 2011). Thus, the pre-ischemic rapid induction of p38 phosphorylation by aldosterone preconditioning could be beneficial for cardiac functional recovery after IRI. In fact, in this, the inhibition of the p38 activity during aldosterone preconditioning was found to hinder the improvement in LV functional recovery after IRI (Fig. 6). In contrast, p38 phosphorylation has been shown to be reduced by short-term aldosterone stimulation in NRCMs (Karmazyn *et al.* 2003), indicating the possibility that aldosterone preconditioning attenuates the maladaptive p38 activation induced by IRI.

The 27-kDa small heat-shock protein (HSP27) is a major downstream regulator of p38MAPK signaling cascades, whose phosphorylation has been shown to protect against IRI (Sanada *et al.* 2001, Marais *et al.* 2005). However, HSP27 phosphorylation levels were not constantly elevated at any point of IRI in this study (data not shown). Although it is difficult to definitively establish causality, our results, together with those of previous studies by others (Mocanu *et al.* 2000, Marais *et al.* 2001, 2005, Sanada *et al.* 2001), revealed that the transient activation of p38 during preconditioning has a trigger action, while the deactivation of p38 after the onset of sustained ischemia–reperfusion acts as a mediator of cardioprotection. Further studies are required to explore the MR-independent aldosterone–p38 signaling pathways and p38-dependent downstream signaling cascades in more detail, including the pathways involved in HSP27 and/or ATF, and elucidate the precise regulatory mechanisms underlying the conversion of the dual activity of p38 induced by aldosterone preconditioning.

Overall, this study highlights the pathophysiological significance of the rapid induction of the cardiac aldosterone cascade in response to critical cardiac conditions, especially during the acute phase. We previously reported increased levels of aldosterone production/secretion from failing human hearts. Moreover, our recent study demonstrated that there is a transient decrease in serum potassium level during ischemic attacks of acute coronary syndrome, which is at least partially mediated through the activation of the aldosterone cascade (Sekiyama *et al.* 2013). Although these clinical findings provide evidence for local cardiac aldosterone synthesis/actions, this issue remains very controversial, and in fact, some reports have offered negative findings regarding the question of local aldosterone synthesis in the heart (Gomez-Sanchez *et al.* 2004, Fiebelor *et al.* 2005, Chai *et al.* 2010). In any case, the results of the current study confirm the speculation that, while aldosterone generally has little effect on the cardiovascular system under baseline normal conditions (Oliver *et al.* 1975), an elevated local/systemic aldosterone level (even within the physiological range, from the low-normal range to the high-normal or just above the normal range, which does not generally exceed a pharmacological dose of over 10^{-8} mol/l) in rapid response to severe cardiac conditions exerts substantial effects on cardiac function, which initially act in a cardioprotective manner. Future studies using aldosterone synthase inhibitors in *in vivo* models of IRI will contribute significantly to defining the critical role of the induction of the pre-ischemic aldosterone cascade,

which actually ‘preconditions’ the heart for subsequent serious of cardiac events. Altogether, this present study provides new insights into the physiological compensatory role of aldosterone under severe cardiac pathological conditions, while still emphasizing the usefulness of MR inhibitors for suppressing unfavorable MR-dependent actions, without blocking favorable MR-independent actions.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-14-0067>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

T Y and T N designed, performed researched, and wrote the paper; R A and Y K performed research and data interpretation; K I, D K, M F and Y K performed research and collected the data; T D and K H analyzed and interpreted data M Y designed, and conducted the study, and wrote the paper.

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