Low-Dose Spironolactone Prevents Apoptosis Repressor With Caspase Recruitment Domain Degradation During Myocardial Infarction

Thi Yen Loan Le, Mahidi Mardini, Viive M. Howell, John W. Funder, Anthony W. Ashton, Anastasia S. Mihailidou

Abstract—Low-dose mineralocorticoid receptor antagonists reduce morbidity and mortality in patients with heart failure and myocardial infarction, despite normal plasma aldosterone levels. Since apoptosis plays an important role in heart failure and postinfarction left ventricular remodeling, we examined whether low-dose mineralocorticoid receptor antagonists modulate cardiomyocyte death by regulating the apoptosis repressor protein apoptosis repressor with caspase recruitment domain to lessen the extent of apoptosis. Hearts from adult male Sprague-Dawley rats were subjected to regional ischemia followed by reperfusion ex vivo, with mineralocorticoid receptor antagonists added to perfusates before ischemia. Low-dose spironolactone (10 nmol/L) or eplerenone (100 nmol/L) significantly reduced infarct size. Spironolactone also prevented cleavage of the apoptotic chromatin condensation inducer in the nucleus and of the inhibitor of caspase-activated DNase induced by ischemia-reperfusion, thereby abolishing chromatin condensation and internucleosomal cleavage. Ischemia-reperfusion–induced activation of caspases 2, 3, and 9, but not caspase 8, was prevented by spironolactone, suggesting targeted regulation of the intrinsic pathway. Low-dose spironolactone and eplerenone prevented loss of the apoptosis repressor with the caspase recruitment domain and reduced myocyte death. In H9c2 cells, mineralocorticoid receptor activation by aldosterone resulted in apoptosis repressor with caspase recruitment domain degradation and enhanced apoptosis; these actions were prevented by coadministration of spironolactone. Using a triple lysine mutant we identified that aldosterone enhances posttranscriptional degradation of the apoptosis repressor with a caspase recruitment domain via the ubiquitin-proteasomal pathway. Our data demonstrate that low-dose mineralocorticoid receptor antagonists reduce infarct size and apoptosis in the reperfused myocardium by preventing the apoptosis repressor with caspase recruitment domain degradation. *(Hypertension. 2012;59:1164-1169.)* ● Online Data Supplement

Key Words: myocardial infarction ▪ apoptosis ▪ ischemia ▪ reperfusion injury ▪ heart failure ▪ cardiovascular disease

M ortality and morbidity after acute myocardial infarction remain high, despite rapid reperfusion by percutaneous coronary intervention, and additional therapeutic strategies that reduce infarct size are needed.1 Ischemia alters redox state triggering apoptosis and progressive loss of cardiomyocytes during and after myocardial infarction, which, in turn, contributes to developing congestive cardiac failure.2 Low-dose mineralocorticoid receptor (MR) antagonists have been shown clinically to reduce blood pressure,3,4 particularly in resistant hypertension, and to substantially increase survival in heart failure,5 heart failure after myocardial infarction,6 and chronic systolic heart failure with mild symptoms.7 Significant benefits follow when eplerenone is administered early (<7 days) after myocardial infarction8 and experimentally at reperfusion.9,10 Interestingly, MR antagonists added to standard treatment proved effective despite levels of plasma aldosterone in the physiological range. In this regard, we have shown recently that low-dose spironolactone (10 nmol/L) perfused alone is protective during ischemia-reperfusion (I-R) in rats, reducing infarct size and apoptosis, and this protective effect persisted in ex vivo I-R studies in hearts from adrenalectomized animals,11 raising interest in defining the mechanism for the cardioprotective action of low-dose MR antagonists.

Received December 25, 2011; first decision January 18, 2012; revision accepted March 20, 2012.

Funding bodies had no role in study design, data collection and analysis, decision to publish, or preparation of the article.

A.W.A. and A.S.M. are shared senior authors for this article.

The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.111.190488/-/DC1.

Correspondence to Anastasia S. Mihailidou, Department of Cardiology, Royal North Shore Hospital, Pacific Highway, St Leonards, Sydney, New South Wales, Australia 2065. E-mail anastasia.mihailidou@sydney.edu.au

Hypertension is available at http://hyper.ahajournals.org

DOI: 10.1161/HYPERTENSIONAHA.111.190488
Apoptosis, or programmed cell death, is a tightly regulated process, accounts for a significant part of the infarct size during myocardial I-R, and plays an important role in left ventricular remodeling. The molecular mechanisms in apoptosis involve initiator caspase activation, an integrative phase and an execution phase involving caspases and endonucleases, apoptotic body formation, and cell fragmentation. Activation of effector caspases occurs via the extrinsic (activated by death receptor ligands) or the intrinsic pathway (involving the mitochondria and endoplasmic reticulum).

Each pathway is characterized by a unique signature of caspase activation.

Interfering with the apoptotic pathway attenuates myocardial injury. Whether low-dose MR antagonists modulate the onset of apoptosis during reperfusion injury has not been investigated previously. Apoptosis repressor with caspase recruitment domain (ARC) is an endogenous apoptosis repressor protein that is highly expressed in cardiac tissue and is unique in its ability to inhibit both death receptor (extrinsic) and mitochondrial (intrinsic) death pathways. Loss of ARC predisposes cardiac myocytes to undergo apoptosis during myocardial stress and mitochondrial remodeling. Decreased ARC expression has been measured in hypertensive animals and myocardium from patients with heart failure. Our aim was to determine whether MR antagonists modulate cardiomyocyte death by regulating apoptosis repressor ARC and preventing the onset of apoptosis.

Methods
Details of materials and methods are provided in the online-only Data Supplement.

Animal Studies
Male Sprague-Dawley rats (N=122; 300–400 g) were used, and study protocols were approved by the Royal North Shore Hospital Animal Care and Ethics Committee. All of the experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Details for ex vivo I-R of the rat heart, measurement of apoptosis, immunohistochemistry, and immunoblotting are available in the online-only Data Supplement.

Cell Culture Studies
Rat cardiomyocyte H9c2 cells were used. Details for the cell culture studies, including routine culture, transfection and generation of cells lines with stable expression or knockdown of ARC, simulated ischemia and dose-response studies are available in the online-only Data Supplement.

Figure 1. Mineralocorticoid receptor (MR) antagonists reduce myocardial infarct size. Various concentrations of spironolactone (SPIRO) and eplerenone (EPL) treatment on infarct size. Treatment with flutamide (Flut; 1 µmol/L) on the effect of SPIRO was also investigated. Infarct area/size (IA) is expressed as percentage of area at risk (AAR). I-R indicates ischemia-reperfusion. Numbers of animals in each group are indicated in parentheses. Values are expressed as mean±SE. *P<0.05 vs I-R alone; #P<0.05 vs sham I-R.

Statistical Analysis
Results are presented as the mean±SE. Statistical comparisons were by unpaired Student t test or for comparing differences between treatments, 1-way ANOVA followed by Holm-Sidak post hoc test to adjust for multiple comparisons. Values of P<0.05 are considered statistically significant.

Results
Low Doses of MR Antagonists Reduce Infarct Size by Preventing Apoptosis
Our dose-response studies confirmed that 10 nmol/L of spironolactone is the lowest dose to significantly reduce infarct size (Figure 1). Although spironolactone is also an androgen receptor antagonist, we found that flutamide (1 µmol/L) did not prevent the protective effect of spironolactone (Figure 1). The selective MR antagonist eplerenone, at 100 and 1000 nmol/L, also significantly reduced infarct size (Figure 1), confirming an MR-mediated effect of spironolactone on infarct size. Exacerbated reperfusion injury leads to increased apoptosis in the area at risk; Figure S1A (in the online-only Data Supplement) shows a representative photomicrograph for colocalization of desmin (green) and TUNEL (red) staining, confirming cardiomyocyte apoptosis. Consistent with the effect on infarct size, I-R produced significant myocardial DNA cleavage measured by TUNEL. Both spironolactone and eplerenone attenuated apoptotic index (Figure S1B) in the reperfused myocardium, similar to their effect on infarct size.

Low Doses of MR Antagonists Modify Nuclear Remodeling Substrates
A apoptotic chromatin condensation inducer in the nucleus (ACINUS) and inhibitor of caspase-activated DNAse (ICAD) are essential caspase substrates for DNA cleavage and apoptotic chromatin remodeling. Using a dual immunofluorescence assay to monitor ACINUS activation, we found that ACINUS was not processed during sham I-R (Figure S2, equal red [N-terminus ACINUS] and green [cleavable C-terminus] staining); however, during reperfusion, 50% of ACINUS was processed (Figures S2 [loss of green staining]) and 2. Low-dose (10 nmol/L) spironolactone prevented ACINUS processing (Figure S2 [equal amounts of red and green staining]) without altering overall ACINUS expression (Figure 2). Similar results were observed with ICAD processing. Reperfusion injury markedly reduced immunoreactivity for full-length ICAD (Figure 2), indicating caspase-mediated...
In contrast, spironolactone prevented I-R–induced loss of XIAP by 10 nmol/L of spironolactone (Figure 3B).

Antiapoptotic proteins Bcl-2, XIAP, and ARC are known to regulate activation of the intrinsic pathway and the associated apoptosome,23,24 and Figure 3A shows representative immunoblots of antiapoptotic proteins, Bcl-2; XIAP, and ARC expression during I-R. Actin was used to control for loading. Analysis shows that ARC protein expression decreased during I-R and was prevented by 10 nmol/L of spironolactone (SPIRO). ARC indicates apoptosis repressor with a caspase recruitment domain; I-R, ischemia-reperfusion; (N=3–6 per group); *P<0.05 vs I-R alone; #P<0.05 vs sham I-R.

Low-Dose MR Antagonist and Proapoptotic Proteins

Caspase 3 mediates cleavage of nuclear substrates ACINUS and ICAD.21,22 During I-R injury, the 32-kDa full-length caspase 3 was cleaved to a 17-kDa fragment shown by staining with a cleavage-specific antibody (Figure S3A) and cleavage of procaspase 3 (Figure S3B). Low-dose (10 nmol/L) spironolactone abolished I-R–induced caspase 3 cleavage (Figure S3A and S3B). Myocardial reperfusion injury also triggered processing of caspase 2 and 9, as indicated by the appearance of the cleaved fragments, and decreased levels of procaspase 2 and 9 proteins (Figure S3C).

In contrast, caspase 8 activity did not change during I-R. Low-dose spironolactone prevented reperfusion-induced activation of caspase 2 and 9 (Figure S3C). Spironolactone prevents reperfusion-induced ARC cleavage.

Low-Dose MR Antagonist Prevents Degradation of Apoptosis Repressor Protein ARC

Antiapoptotic proteins Bcl-2, XIAP, and ARC are known to regulate activation of the intrinsic pathway and the associated apoptosome.23,24 and Figure 3A shows representative immunoblots. I-R downregulated Bcl-2 and XIAP by >50%, with neither affected by 10 nmol/L of spironolactone (Figure 3B).

In contrast, spironolactone prevented I-R–induced loss of ARC expression (~60% to 80%; Figures 3B and S4). Eplerenone produced a similar response to spironolactone, restoring levels of ARC in reperfused hearts (Figure S4). Therefore, low-dose MR antagonists prevent apoptosis during reperfusion injury by maintaining ARC levels, resulting in reduced infarct size.

To investigate whether salvage of ARC expression was essential for the antiapoptotic effects of spironolactone, we transfected H9c2 cells with an ARC knockdown construct (short hairpin RNA). ARC expression was abolished by the ARC short hairpin RNA construct (Figure S5B). Cells were then subjected to simulated ischemia and apoptotic index measured. Simulated ischemia markedly reduced ARC levels (Figure S5C) and increased apoptotic index (Figure S5D); low-dose spironolactone restored ARC levels (Figure S5C) and significantly reversed ischemia-induced apoptosis (Figure S5D). In ARC-short hairpin RNA–transfected cells, ischemia-induced apoptosis was exacerbated (Figure S5D), indicating that residual ARC could not effectively oppose apoptosis. In contrast to nontransfected cells, spironolactone did not salvage the induction of apoptosis because of simulated ischemia in short hairpin RNA–transfected H9c2.

MR Activation Promotes ARC Degradation to Initiate Apoptosis In Vitro

The above data suggest that ARC is targeted for degradation on MR activation during reperfusion. Using H9c2 cells, we...
found that MR activation decreased ARC protein levels by \( \approx 45\% \) in vitro (Figure 4A; for optimal dose and exposure times see Figure S6). This effect was prevented by spironolactone (Figure 4A), indicating that it was MR mediated. In contrast, ARC transcription measured by RT-PCR remained unchanged during treatment (Figure 4B), indicating post-translational regulation of ARC levels. ARC contains lysines at positions 17, 68, and 163, and expression is regulated by proteosomal degradation. Mutation of all 3, lysine to arginine (ARC-K3R), renders ARC resistant to degradation, providing a ubiquitination-deficient mutant of the ARC protein. H9c2 cells transfected with ARC mutant (ARC-K3R) were incubated in serum-containing medium (\( \pm \)alosterone, 10 nmol/L). MR activation reduced endogenous ARC protein levels (H9c2 and control vector; Figure 4C) and expression of wild-type human ARC-transfected H9c2 cells. Conversely, ARC-K3R mutant expression levels did not change (Figure 4C), indicating that MR activation leads to ARC degradation through the ubiquitin-proteosomal pathway.

**Discussion**

Our study identifies novel mechanisms of action of low-dose MR antagonists on cardiomyocytes during myocardial infarction, preventing degradation of the antiapoptotic protein ARC and processing of the death-promoting proteins, ACINUS and ICAD. They also provide insight into how MR-regulated signaling during reperfusion injury leads to enhanced myocardial apoptosis and left ventricular remodeling and why low-dose antagonists (eg, spironolactone) may be so effective for the treatment of heart failure and associated conditions where low-level apoptosis drives cardiac damage and remodeling.

Restoration of ARC expression may explain the ability of MR antagonists, such as spironolactone and eplerenone, to regulate activation of the intrinsic pathway during reperfusion injury. Activation of MR during reperfusion injury targets ARC for posttranslational degradation through the ubiquitin-proteosomal pathway. This event is not only critical for apoptosis progression but also an important indicator of the potential effectiveness of MR antagonist treatment. Spironolactone cannot prevent myocyte apoptosis in the absence of ARC expression. Therefore, ongoing suppression of Nol3 transcription or diminished ARC protein half-life would limit MR antagonists as therapeutic options. Understanding the mechanism by which low-dose MR antagonists prevent ARC degradation provides potential for developing therapeutic regimens in the treatment of heart failure and myocardial infarction. Currently, low doses of MR antagonists are used in both heart failure and heart failure post-MI.

Our ex vivo I-R model results in basal measures of myocardial infarct size comparable to previous studies, during in vitro and in vivo I-R. The protective effect of spironolactone persisted in the presence of flutamide, indicating MR-mediated reduction in infarct size. The responses that we observed at the higher doses of MR antagonists are comparable to a previous study, under the same experimental conditions. In contrast, another study showed no effect of either eplerenone at 1 \( \mu \)mol/L or canrenone at 10 \( \mu \)mol/L, whereas in vivo studies using 80 to 100 mg/kg per day of spironolactone showed only a 1% reduction in infarct size. To our knowledge, there has only been one other study that has determined the effect of a low dose of spironolactone (10 nmol/L) during I-R. Although infarct size was not measured in that study, low-dose spironolactone (10 nmol/L) significantly increased contractility.

Increased apoptotic rate during human acute myocardial infarction leads to progression of heart failure, and, therefore, alteration of apoptosis pathways may attenuate myocardial injury. Aldosterone induces cardiomyocyte apoptosis, although the specific pathway was not identified, with both MR-dependent and -independent pathways proposed. Although Akt and extracellular signal-regulated kinase 1/2 activation have been shown to be molecular targets for higher doses of MR antagonists during I-R, apoptosis was not measured. Excluding our previous study only 3 other studies explore the effect of MR antagonists on apoptosis. In 1 of these studies apoptosis was assessed only by TUNEL after 100 mg/kg per day of spironolactone. Our studies in H9c2 cardiomyocytes complement our studies in reperfused hearts and show a direct effect of MR activation on steady-state levels of ARC. We used H9c2 cells as a controlled environment to identify whether ARC regulation by low-dose MR antagonists was MR dependent rather than an MR-independent action. Under normoxic conditions and using serum-containing medium, we show that MR activation by
aldosterone decreases ARC protein levels, independent of simulated ischemia. Under similar normoxic conditions, cortisol would be without effect. These results are supported by previous studies that propose that the ratio of death inducer to death repressor proteins exerts control over cell survival. Myocardial apoptosis, both at the site of infarction and in the remote zone, correlates with unfavorable postinfarction left ventricular remodeling and progression to heart failure.

Our results provide a possible mechanism for the cardioprotective action of low-dose MR antagonists in the clinical studies (Randomized Aldactone Evaluation Study and Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study) to regulate cardiomyocyte ARC and to reduce this ongoing low-grade myocardial apoptosis.

Nuclear remodeling during apoptosis requires activation of cysteinyl-aspartate proteases (caspases), located in the cell as inactive proenzymes (upstream initiator caspases; eg, procaspases 2, 8, 9, 10, and 12) and downstream effector caspases (eg, procaspases 3, 6, and 7) mediating unwinding of DNA and subsequent internucleosomal cleavage. Spironolactone attenuating I-R–induced processing of caspase 3 (and subsequent cleavage of nuclear substrates and nuclear fragmentation) is almost certainly a product of inactivation of upstream pathways. In our studies, processing of caspase 2 and 9 (both involved in the intrinsic pathway) was indicated by the appearance of the cleaved fragments and decreased levels of procaspase 2 and 9 proteins (Figure S3C). In contrast, procaspase 8 activity (involved in the extrinsic pathway) did not change during I-R. Low-dose spironolactone prevented activation of procaspase 2 and 9 (Figure S3C) during I-R, confirming that MR antagonists regulate the intrinsic apoptosis pathway.

Our study confirms that antiapoptotic proteins Bcl-2, XIAP, and ARC must be degraded for apoptosis to proceed during myocardial I-R injury. MR antagonist-induced stabilization of ARC expression prevents activation of the intrinsic pathway at multiple points. ARC inhibits t-Bid generation and caspase 2 processing by the PIDDosome through interaction between the CARD domains of the 2 proteins and prevents Bax activation in response to I-R injury. There have been discrepant reports for activation of the death receptor (extrinsic) pathway during I-R, with both the absence of caspase 8 processing noted, confirming our findings, and activation reported by other studies.

ARC is targeted via ubiquitination to the proteasome and is unique compared with other antiapoptotic proteins that act on only 1 apoptotic pathway. Preserving ARC levels is sufficient to maintain myocyte viability after oxidant stress or infarction. Mutation of ubiquitin acceptor sites on ARC prevents degradation and enhances cellular protection, confirming the role of decreasing ARC levels as the master controller of cardiomyocyte apoptosis. Regulation of ARC expression during reperfusion is controlled by the expression/activity of the ubiquitin ligase MDM2. MR activation upregulates MDM2, leading to proliferation of vascular smooth muscle cells and, as shown in our studies, degradation of ARC. MDM2 is also the ligase for p53, controlling both ubiquitination and degradation of p53. MR activation regulates p53, a transcriptional regulator of the proapoptotic protein Bax. ARC interacts with the C-terminal regulatory domain of Bax, preventing conformational activation and translocation from the cytosol to the mitochondria, leading to decreased apoptosis, further confirming that the mitochondrial (or intrinsic) apoptotic pathway is involved.

**Perspectives**

In conclusion, our findings show that low-dose MR antagonists regulate cardiomyocyte apoptosis repressor protein ARC during I-R. This novel action of MR antagonists provides direct evidence that antagonists have effects beyond denying agonists access to MR, so-called MR blockade. The low doses of spironolactone (and lower affinity of MR-selective antagonist eplerenone) prevented ARC degradation, providing cardioprotection by preventing initiation of apoptosis during myocardial infarction and, by extension, in hypertension, where they are increasingly used. Our studies support early administration of low-dose MR antagonists or addition to drug-eluting stents for protection of the myocardium.

**Acknowledgments**

We thank Prof Celso Gomez Sanchez for providing the MR antibodies and Prof Richard Kitsis for the K,R-ARC-HA construct.

**Sources of Funding**

This study was supported by a project grant from the National Heart Foundation of Australia (A.S.M.). T.Y.L.L. was supported by postgraduate fellowship (633112) and A.W.A. by a Career Development Award (402847) from the National Health and Medical Research Council and V.M.H. by a Cancer Institute New South Wales Fellowship.

**Disclosures**

J.W.F. is a consultant for Pfizer, Merck, Novo Nordisk, and Allergan, as well as a member of the Board of CBio. All other authors have no conflicts of interests to disclose.

**References**


32. Mihailidou AS, Mardini M, Fraser T, Funder JW. Agonist/antagonist activity of cortisol in cardiomyocyte mineralocorticoid receptors is determined by redox state. Presented at the 30th Meeting of the International Aldosterone Conference; June 14–15, 2004; New Orleans, LA.


47. Sohn HJ, Yoo KH, Jang GY, Lee JH, Choi BM, Bae IS, Yim HE, Son CS, Loan Le et al MR Antagonists and ARC
Low-Dose Spironolactone Prevents Apoptosis Repressor With Caspase Recruitment Domain Degradation During Myocardial Infarction

Thi Yen Loan Le, Mahidi Mardini, Viive M. Howell, John W. Funder, Anthony W. Ashton and Anastasia S. Mihailidou

Hypertension. 2012;59:1164-1169; originally published online April 16, 2012;
doi: 10.1161/HYPERTENSIONAHA.111.190488

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/59/6/1164

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2012/04/16/HYPERTENSIONAHA.111.190488.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
LOW DOSE SPIRONOLACTONE PREVENTS ARC (APOPTOSIS REPRESSOR WITH A CASPASE RECRUITMENT DOMAIN) DEGRADATION DURING MYOCARDIAL INFARCTION

Le: MR antagonists and ARC

Thi Yen Loan Le¹,² BMedSc, Mahidi Mardini¹,²,³ MBBS FRACP PhD, Viive M Howell²,⁴ BSc(App) MSc, PhD, John W Funder⁵, MD PhD FRACP, Anthony W Ashton²,⁶ BSc PhD* and Anastasia S. Mihailidou¹,² BSc PhD*

¹Department of Cardiology, Royal North Shore Hospital, ²Kolling Institute of Medical Research, Royal North Shore Hospital and The University of Sydney; ³Department of Cardiology, Westmead Hospital, ⁴Hormone and Cancer Division, ⁵Prince Henry’s Institute, Clayton, Victoria, ⁶Division of Perinatal Research, Royal North Shore Hospital, Australia.
Expanded Methods

Materials: Drugs and chemicals were purchased from Sigma–Aldrich unless otherwise specified. Cell culture reagents were from Invitrogen.

Ex vivo ischemia-reperfusion of the rat heart

Rats were anesthetized with intraperitoneal (i.p.) ketamine (60 mg/kg) and xylazine hydrochloride (10 mg/kg), and then received heparin (250 IU, i.p.). Hearts were rapidly isolated and mounted onto a Langendorff apparatus, with regional I-R induced by occluding a branch of the left coronary artery for 30 min and releasing the occlusion to allow reperfusion for 150 min, as previously described [1-3]. MR antagonists, spironolactone (SPIRO, 1, 3, 10 and 1000 nM) or eplerenone (EPL, 100 and 1000 nM) were perfused for 15 minutes prior to ischemia and throughout reperfusion. We used a higher initial dose of eplerenone (100 nM) since affinity of eplerenone in vitro is 2-3% that of spironolactone [4]. In separate experiments, the androgen receptor antagonist, flutamide (1 μM) was administered alone or with spironolactone. At the end of reperfusion, hearts were infused with monastral blue dye to delineate area at risk, and infarct size measured as described previously [3]. Infarct size, apoptosis and immunohistochemistry were measured on the same hearts, with a separate group of animals used for western blot analysis.

Measurement of apoptosis in cardiac tissue

Apoptosis was measured by terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) as previously described [3]. Immunostaining for Desmin was used to confirm cardiomyocyte apoptosis [5]. Following TUNEL staining, desmin antibody (10%, Abcam) was applied to sections of reperfused left ventricle and left overnight at 4°C before counterstaining with DAPI reagent. Images were captured using fluorescence microscopy at 200x magnification.

Immunohistochemistry

Immunostaining for apoptotic mediators was performed using formalin-fixed paraffin embedded sections of reperfused left ventricle (5 μm thick). Sections were deparaffinised, rehydrated and endogenous peroxidase activity blocked by incubation with 1% hydrogen peroxide for 30 min and epitope retrieval performed in 10 mM citrate buffer (pH 6.0) at 95°C for 10 min. Sections were immunostained for apoptotic mediators using specific antibodies against ARC (Cayman), active caspase-3 (CM1, Abcam), and by dual immunofluorescence for ACINUS using C- (AnaSpec) and N-(Santa Cruz) terminal and specific secondary antibodies. For caspase-3 and ACINUS staining antibody reactivity was detected using either FITC- or Cy3-labelled secondary antibody (1:200) before counterstaining with DAPI reagent. For all other antibodies reactivity was detected using envision+ polymer reagent (DAKO) with DAB used as the chromogen (Sigma-Aldrich). Images were captured using either bright field or fluorescence microscopy (200x magnification).

SDS-PAGE and Immunoblotting

At the end of reperfusion, left ventricular free wall tissue was snap frozen in liquid nitrogen. For protein extraction, frozen tissue was powdered in a mortar and pestle and homogenized in RIPA buffer (50 mM Tris (pH8.0), 150 mM NaCl, 1% (v/v) Triton x100, 1% (w/v) Na-deoxycholate, 0.1% (w/v) SDS, 1 mM PMSF, and protease inhibitors). Lysates were immediately placed into on ice for 30 minutes at 4°C followed and clarified by centrifugation at 16,000xg for 10 min. Protein content of lysates was measured by Bradford Protein Assay [6]. Immunoblotting was performed as previously described [7]. Aliquots (30-50 μg) were
separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on gels of varying acrylamide concentrations (8-15 %). Following transfer to PVDF membranes, primary antibodies including ACINUS (C-terminal), ICAD (N-terminus), caspase-3/-2/-8/-9, ARC, B-cell lymphoma (Bcl)-2 and X-linked inhibitor of Apoptosis (XIAP) were diluted in blocking buffer (TBS-T (10 mM Tris (pH8.0), 150 mM NaCl, 0.1% (v/v) Tween-20) and 5% (w/v) non-fat milk powder) and incubated with membranes overnight at 4°C. Antibody binding was detected with HRP-conjugated secondary antibodies (1:3000, Dako Cytomation, Dako Australia). Membranes were washed, exposed to chemiluminescent substrate (Perkin Elmer, Melbourne, Australia) and digital images of the resulting bands captured on a LAS4000 (GE Healthcare Life Sciences).

**Cell culture studies**

To determine whether the mechanism for the cardioprotective action of low dose MR antagonists required regulating ARC processing, we used the rat cardiomyocyte cell line (H9c2) since many of the characteristics of isolated primary cardiomyocytes are retained [8, 9]. H9c2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) which contained 10% fetal bovine serum, L-glutamine (2 mM), penicillin (10 U/mL) and streptomycin (10 μg/mL) at 37°C in a humidified atmosphere with 5% CO₂ and at ~90% confluence were harvested for experiments. Cells were treated with this medium for all studies except during the simulated ischemia protocol.

**Simulated ischemia and apoptosis studies in H9c2 cells**

Simulated ischemia was achieved by culturing cells in serum free-, glucose free-DMEM in an atmosphere of 1% (v/v) O₂/5% (v/v) CO₂/94% (v/v) N₂ at 37°C for 18 h. For detecting apoptosis, cells were fixed in 4% (w/v) paraformaldehyde in PBS and permeabilized with 0.1% (v/v) Triton X-100 in 0.1% (w/v) sodium citrate, followed by incubation in TUNEL reaction mixture for 60 min at 37°C. After washing, the cells were counterstained with DAPI, mounted and visualised under fluorescence microscope. TUNEL-positive nuclei were calculated in five randomly selected fields for each slide and three independent experiments were used per treatment.

**Dose response and time-course studies for aldosterone in H9c2 cells**

The cells were plated in 6 well plates and allowed to adhere overnight in complete media. The effective dose (EC50) of aldosterone was determined by treating cells with 1, 2, 5, 10 and 20 nM aldosterone for 24 h prior to harvest. For the time-course study, cells were treated with 10 nM aldosterone and harvested at 6, 12, 18, 24 and 36 h. In separate experiments, optimal conditions for aldosterone stimulation were used to examine the selectivity of MR-activation in the observed effects. H9c2 cells were pre-incubated with 100 nM spironolactone for 30 min prior to aldosterone addition. Cells were then treated with aldosterone ± spironolactone. Treated cells were washed with PBS, trypsinized, and then resuspended in 200 µL of lysis buffer (50 mM Tris (pH8.0), 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.02% (w/v) sodium azide, and protease inhibitors) and protein extracted as described above.

**Studies using ARC-knockdown construct**

To permanently knockdown ARC, H9c2 cells were transfected with shRNA constructs against ARC and a scramble control (Open Biosystems). Selection was performed using 2 μg/mL puromycin (Sigma Aldrich) and pooled populations used in subsequent experiments. Stably transfected H9c2 cells were screened for ARC expression by immunoblotting to confirm extent of knockdown.
Studies using a ubiquitination-resistant ARC mutant
H9c2 cells were transfected with expression vectors for wild-type (hARC-HA) and a ubiquitination deficient mutant (hARC-K3R-HA) [10] of ARC using Effectene according to the manufacturer’s protocol (Qiagen). An empty vector (pcDNA3.1) was used as a control. Cell lines with stable overexpression were generated using antibiotic selection (150 µg/mL hygromycin) (Sigma Aldrich) for 2 weeks and pooled populations used in subsequent experiments. Expression of the constructs was confirmed by immunoblotting for the HA tag (Santa Cruz). Cells were then incubated with serum and glucose-containing media ± 10 nM aldosterone for 24 h and protein levels were determined by western blot.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)
Total RNA was extracted from H9c2 cells using Qiazol lysis reagent (Qiagen) and 5 µg was reverse transcribed using the Superscript III First-Strand Synthesis System for RT-PCR kit (Invitrogen) and oligo (dT) according to the manufacturers’ protocols. The Nol3 gene that encodes ARC was amplified with primers: Nol3-exon1-F: 5’-CTACTGCTGTGGTGCA-3’ and Nol3-exon2-R: 5’-GACCTCCGATCTCCTCTTCC-3’, resulting in a 236bp product. The endogenous control Gusb (glucuronidase beta) was amplified with primers: Gusb-exon3-F: 5’-GGTGTGGTATGACGGAAG-3’ and Gusb-exon4-R: 5’-TGGTGATGTCAGCCTCAAAG-3’, resulting in a 170bp product. Amplification was carried out for 30 cycles in a 50 µL volume with 2 µL cDNA and the products were separated using electrophoresis on 2% (w/v) TAE-agarose gels and visualised, after staining with Gel red solution, using UV light on a gel imaging system (GE Healthcare Life Sciences).

References

**Supplement Figures**

**Figure S1. MR antagonists attenuate apoptosis during ischemia-reperfusion.**

A. Representative photomicrographs of ventricular sections show proportion of TUNEL-positive nuclei (red fluorescence, arrows) relative to DAPI (blue fluorescence) stained nuclei. Desmin antibody (green fluorescence) indicates cardiomyocytes. Arrows indicate apoptotic cardiomyocyte nuclei (pink). All images are x200 magnification. B. Quantification of the apoptotic index from treated reperfused myocardium. I-R, ischemia-reperfusion, SPIRO, spironolactone, EPL, eplerenone. Values express as mean ± SE. Numbers in parentheses indicate number of animals in group. *p<0.05 vs I-R alone; #p<0.05 vs sham I-R.

**Figure S2: Spironolactone prevents ACINUS processing**

A. Dual immunofluorescence staining for ACINUS identifying the non-activation dependent epitope (N-terminus, red) and proteolytically processed C-terminus (green). The two fluorophores co-localize (yellow fluorescence) when ACINUS is inactive. Loss of green fluorescence is observed when ACINUS is cleaved by effector caspases in the reperfused myocardium. Low dose spironolactone (SPIRO 10 nM) prevented ACINUS processing.
Figure S3. Spironolactone attenuates apoptosis by preventing activation of the intrinsic pathway during IR injury.

A. Myocardial sections stained for active caspase-3. Increased green fluorescence indicates cleavage of caspase-3; spironolactone (SPIRO 10 nM) prevented caspase-3 processing. B and C. Representative immunoblots showing processing of pro-caspases-3 (B), -2, -8, and -9 (C) in lysates from reperfused left ventricular tissue samples compared to sham I-R. SPIRO prevented cleavage of caspases-3, -2 and -9. No changes in pro-caspase-8 protein levels were observed. I-R, Ischemia-reperfusion, FL, full-length, (N=3-6 per group). Changes in B and C were quantified using densitometric analysis with Actin used as the loading control. *p<0.05 vs I-R alone; #p<0.05 vs sham I-R.

Figure S4. Low dose MR antagonists prevent reperfusion-induced ARC degradation.
Sections of reperfused myocardium were stained for ARC (apoptosis repressor with a caspase recruitment domain) using 3, 3’ diaminobenzidine (DAB, brown precipitate). Bar graph shows quantitative analysis of ARC expression. I-R, ischemia-reperfusion, SPIRO, spironolactone, EPL, eplerenone. #p<0.05 vs sham I-R; *p<0.05 vs I-R alone.
Figure S5. Direct regulation of ARC by low dose spironolactone during simulated ischemia (hypoxia).

A. Both MR and ARC are expressed in H9c2 cells.  B. ARC expression was abolished by the ARC shRNA construct compared with non-transfected (B) or scrambled shRNA transfected cells (data not shown). C. ARC protein expression in normoxic and hypoxic H9c2 cells was determined by western blotting using Actin as the loading control. ARC levels decreased during hypoxia and were restored by low dose spironolactone in H9c2 cells. D. Quantification of the apoptotic index during simulated ischemia by TUNEL staining. Low dose spironolactone reversed the effects of simulated ischemia in H9c2 cells with intact ARC levels but not in knockdown cells. MR, mineralocorticoid receptor, ARC, Apoptosis Repressor with a Caspase recruitment domain, SPIRO, spironolactone. Representative gel of three experiments and values expressed as mean ± SE. *p<0.05 vs hypoxia alone.

Figure S6: Effect of aldosterone exposure time and dose on ARC expression in H9c2 cells

A. Time course for regulation of ARC expression by 10 nM aldosterone. Processing quantified by densitometry and Actin used to control for loading. Values expressed as mean ± SE, *p<0.05 vs control (t=0). B. Varying doses of aldosterone on ARC expression in H9c2 cells. Experiments were performed in triplicate.