

Novel Cardiac Apoptotic Pathway

The Dephosphorylation of Apoptosis Repressor With Caspase Recruitment Domain by Calcineurin

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Background—Apoptosis repressor with caspase recruitment domain (ARC) is abundantly expressed in cardiomyocytes. Protein kinase CK2 can phosphorylate ARC at threonine-149, thereby enabling ARC to antagonize apoptosis. ARC phosphorylation occurs in a constitutive manner. Nevertheless, cardiomyocytes still undergo apoptosis that is related to cardiac diseases such as myocardial infarction and heart failure. Whether the occurrence of apoptosis is related to the loss of protection by ARC under pathological conditions remains unknown.

Methods and Results—ARC phosphorylation levels are decreased in cardiomyocytes treated with isoproterenol or aldosterone. We explored the molecular mechanism by which ARC phosphorylation levels are decreased. Our results reveal that either direct incubation or coexpression with calcineurin leads to a decrease in ARC phosphorylation levels. Inhibition of calcineurin can attenuate the reduction in ARC phosphorylation levels on treatment with isoproterenol or aldosterone. These data indicate that the reduction in ARC phosphorylation levels is related to its dephosphorylation by calcineurin. Our results further reveal that ARC can prevent isoproterenol- and aldosterone-induced apoptosis, but this function depends on its phosphorylation status. Isoproterenol and aldosterone upregulate Fas ligand expression, and Fas ligand and caspase-8 are required for isoproterenol and aldosterone to induce apoptosis. However, phosphorylated but not dephosphorylated ARC is able to inhibit caspase-8–mediated apoptosis. Phosphorylated ARC exerts its effects against caspase-8 by directly associating with procaspase-8 and inhibiting its interaction with Fas-associated protein with death domain.

Conclusions—Our study identifies a novel cardiac apoptotic pathway in which ARC is dephosphorylated by calcineurin. This pathway could be a component in the cardiac apoptotic machinery. (*Circulation*. 2008;118:2268-2276.)

Key Words: apoptosis ■ calcineurin ■ myocytes, cardiac ■ heart diseases ■ heart failure ■ signal transduction

Apoptosis can occur in the myocardium under a variety of pathological conditions. For example, myocyte apoptosis is increased in myocardium from patients with myocardial infarction and heart failure and from experimental models of hypertrophy and heart failure. In particular, apoptosis plays a driving role in the transition from compensated hypertrophy to failure in the work-overloaded myocardium.^{1,2}

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Apoptosis repressor with caspase recruitment domain (ARC) is abundantly expressed in the heart.³ It can directly bind to caspase-8³⁻⁵ and inhibit caspase-8 activity.^{3,4} ARC inhibits caspase-8 activation by blocking the formation of death-inducing signaling complex. It binds to Fas and Fas-associated protein with death domain (FADD), thereby preventing death-inducing signaling complex formation.⁵ Further studies reveal that ARC also may elicit its function

through other ways. It can interact with Bax, thereby inhibiting Bax activation and translocation to the mitochondria.^{5,6} It inhibits cytochrome c release⁷ and maintains mitochondrial membrane potential.^{8,9} In addition, ARC is regulated by protein kinase CK2 (CK2). CK2 can phosphorylate ARC at threonine-149 (T149). This phosphorylation enables ARC to translocate from the cytoplasm to the mitochondria where it directly binds to caspase-8⁴ or caspase-2.¹⁰ ARC requires T149 phosphorylation to protect cells against oxidative stress–induced apoptosis.¹⁰ Strikingly, ARC phosphorylation by CK2 is constitutive, indicating the importance of phosphorylation for its function.^{4,10}

Despite the abundant expression of ARC, constitutive activation, and multiple ways to inhibit apoptosis, cardiomyocytes still undergo apoptosis under pathological conditions. Cardiomyocyte apoptosis can be triggered by a variety of stimuli such as oxidative stress,⁸ angiotensin II,¹¹ and Fas

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ligand (FasL).¹² Apoptosis is controlled by a complex interplay between proapoptotic and antiapoptotic factors. Under physiological conditions, this interplay remains in balance so that apoptosis is tightly controlled. However, the disruption of this balance under pathological conditions can result in apoptosis. Whether ARC can contribute to this imbalance when it loses its antiapoptotic function remains to be elucidated.

The β -adrenergic receptor agonist isoproterenol promotes cardiac remodeling, including apoptosis.^{13,14} Aldosterone can induce cardiomyocyte apoptosis both in vitro^{15,16} and in vivo.^{15,17} However, the molecular mechanisms by which isoproterenol and aldosterone induce cardiac apoptosis are not completely understood.

Calcineurin is a serine/threonine protein phosphatase. It is composed of a catalytic subunit (calcineurin A [CnA]) and a regulatory subunit (calcineurin B [CnB]). Calcineurin activation can be proapoptotic in cardiomyocytes. For example, coexpression of the constitutively active CnA together with CnB leads to apoptosis in cardiomyocytes.¹⁸ Isoproterenol-induced cardiac apoptosis is related to calcineurin activation.¹⁹ Aldosterone-induced apoptosis also depends on the activation of calcineurin.¹⁶ The ARC phosphorylation site is a threonine residue. It is not yet clear whether calcineurin affects ARC in the apoptotic cascades.

The present work reveals that isoproterenol and aldosterone can induce ARC dephosphorylation through the activation of calcineurin. ARC dephosphorylation leads to its inability to inhibit caspase-8, thereby initiating apoptosis. Our data reveal a novel cardiac apoptotic pathway in which calcineurin dephosphorylates ARC.

Methods

Cell Cultures, Cell Viability Assay, Cell Death Detection ELISA, and Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling Assay

Cultures of neonatal rat cardiac cells, Trypan blue exclusion, cell death detection ELISA, and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay were performed as described elsewhere.^{20,21}

RNA Interference Constructions of Caspase-8, ARC, and FasL

The caspase-8 RNA interference (RNAi) sense sequence is 5'-GCTCTCAGAAGACATGGAC-3'; the antisense sequence is 5'-GTCCATGCTTCTGAGAGC-3'. The scrambled caspase-8 RNAi sense sequence is 5'-GCTGTAGAGACAGACTCAC-3'; the scrambled antisense sequence is 5'-GTGAGTCTGTCTTACAGC-3'. The ARC RNAi sense sequence is 5'-CTAGAAGCTGAAGCTACTA-3'; the antisense sequence is 5'-TAGTAGCTTCAGCTTCTAG-3'. The scrambled ARC RNAi sense sequence is 5'-CTATGAACGAA-TCGATGCA-3'; the scrambled antisense sequence is 5'-TGCATCGATTTCATAG-3'. The FasL RNAi sense sequence is 5'-AAGCAAATAGCCAACCCCA-3'; the antisense sequence is 5'-TGGGGTTGGCTATTTGCTT-3'. The scrambled FasL RNAi sense sequence is 5'-ATCCGAAGCACCACAACAA-3'; the scrambled antisense sequence is 5'-TTGTTGTGGTCTTCGGAT-3'. They were cloned into p*Silencer* adeno 1.0-CMV vector (Ambion).

Adenovirus Constructions and Infection

Adenoviruses were constructed with the Adeno-X Expression System (Clontech Laboratories, Inc, Palo Alto, Calif). They include the wild-type rat ARC (AdwtARC), the rat ARC mutant with T149 converted to an aspartic acid residue (AdARC149D), and the nonphosphorylatable rat ARC mutant with T149 converted to a nonphosphorylatable alanine residue (AdARC149A). Cells were infected at the indicated multiplicity of infection (MOI) for 60 minutes. Under such an infection condition with an MOI of 100, 92.2 \pm 3% of cells overexpress ARC as analyzed by immunofluorescent staining with anti-ARC antibody; 94.6 \pm 2.9% of cells were infected by adenoviruses as analyzed by an adenovirus kit (Imagen, Adenovirus, Dako, Glostrup, Denmark).

Transfection of Cells

Cells were transfected with the plasmid constructs using the Effectene Transfection Kit (Qiagen, Hilden, Germany). Mouse constitutively active CnA (Δ CnA) and CnB cDNA constructs were kindly provided by Dr Thomas Grundström.

Mitochondrial Preparations, Immunoprecipitation, and Immunoblot Analysis

Mitochondrial preparations, immunoprecipitation, and immunoblot were carried out as previously described.²¹ The anti-phospho ARCT149 antibody was from Eurogentec. The anti-ARC antibody was from Chemicon.

Calcineurin Activity Assay and ARC Dephosphorylation by Calcineurin In Vitro

Calcineurin activity was analyzed with the Calcineurin Cellular Assay Kit (Biomol International). ARC dephosphorylation by calcineurin in vitro was performed as described.²² ARC (0.2 μ g) was incubated for 20 minutes at 30°C with the active calcineurin (Biomol International).

Detection of Caspase-8 and CK2 Activity

Caspase-8 activity was detected with an assay kit (R&D Systems, Minneapolis, Minn). CK2 activity was measured as previously described.⁴

Statistical Analysis

Data are expressed as mean \pm SEM. Statistical analyses of parameters of cell death, TUNEL-positive cells, caspase-8 activity, and ARC phosphorylation levels were performed with Student *t* test for comparisons between 2 means. One-way ANOVA with the Tukey post hoc test was used to test for differences in the ability of ARC149D and wild-type ARC (wtARC) to inhibit apoptosis in the presence of 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB). Values of *P* < 0.05 were considered significantly different.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Isoproterenol and Aldosterone Induce a Decrease in ARC Phosphorylation Levels

We first set up an apoptotic model by treating cardiomyocytes with isoproterenol. Isoproterenol treatment could induce apoptosis analyzed by the TUNEL assay (Figure 1A). Cell death ELISA, which specifically detects histone-associated DNA fragments, was used to further confirm whether apoptosis occurred. Oligonucleosomes increased in cells treated with isoproterenol (Figure 1B). Next, we detected ARC phosphorylation levels in cardiomyocytes on isoproterenol treatment. Isoproterenol treatment was able to cause a decrease in ARC phosphorylation levels (Figure 1C).

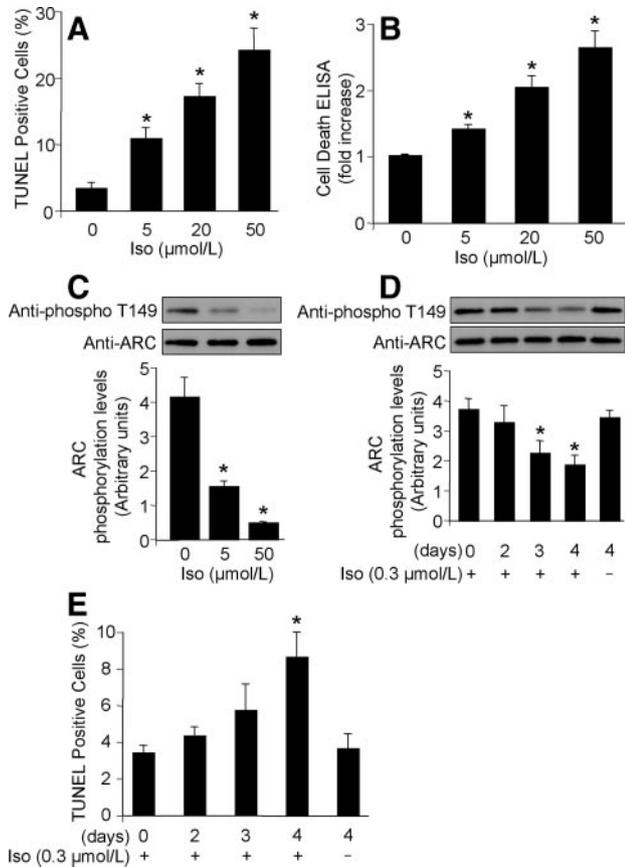


Figure 1. Isoproterenol induces a decrease in ARC phosphorylation levels. Isoproterenol (Iso) induces apoptosis analyzed by TUNEL assay (A) and cell death ELISA (B). Cardiomyocytes were treated for 48 hours with isoproterenol. **P*<0.05 vs control. C, Analysis of ARC phosphorylation levels. Cells were treated for 6 hours with isoproterenol. ARC phosphorylation levels were analyzed by immunoblot using the anti-phospho T149 antibody. Top, A representative blot. Bottom, The results of immunoblot were scanned, and the ratio of ARC protein levels to ARC phosphorylation levels was calculated. **P*<0.05 vs control. D, Sustained treatment with isoproterenol at a low dose leads to a decrease in ARC phosphorylation levels. The culture medium containing isoproterenol was changed once a day. ARC phosphorylation levels were analyzed by immunoblot as described in C. Top, A representative result. Bottom, Quantitative analysis of ARC phosphorylation levels by densitometric scanning. **P*<0.05 vs day 0. E, Sustained treatment with isoproterenol at a low dose leads to apoptosis analyzed by TUNEL assay. Cells were treated as described in D. **P*<0.05 vs day 0. Data in Figure 1 are expressed as the mean±SEM of 3 or 4 independent experiments. All comparisons were performed with Student *t* test.

Sustained treatment with isoproterenol at a low dose induced a decrease in ARC phosphorylation levels (Figure 1D) and an increase in the percentage of apoptotic cells (Figure 1E).

Aldosterone could induce cardiomyocyte apoptosis as revealed by TUNEL (Figure 2A) and cell death ELISA (Figure 2B). ARC phosphorylation levels were decreased on aldosterone treatment (Figure 2C). Sustained treatment with aldosterone at a low dose induced a decrease in ARC phosphorylation levels (Figure 2D) and an increase in the percentage of apoptotic cells (Figure 2E). CK2 activity assay revealed no significant alterations in CK2 activity in cardiomyocytes treated with isoproterenol or aldosterone (data not shown). Thus, these results suggest that the reduction in ARC phos-

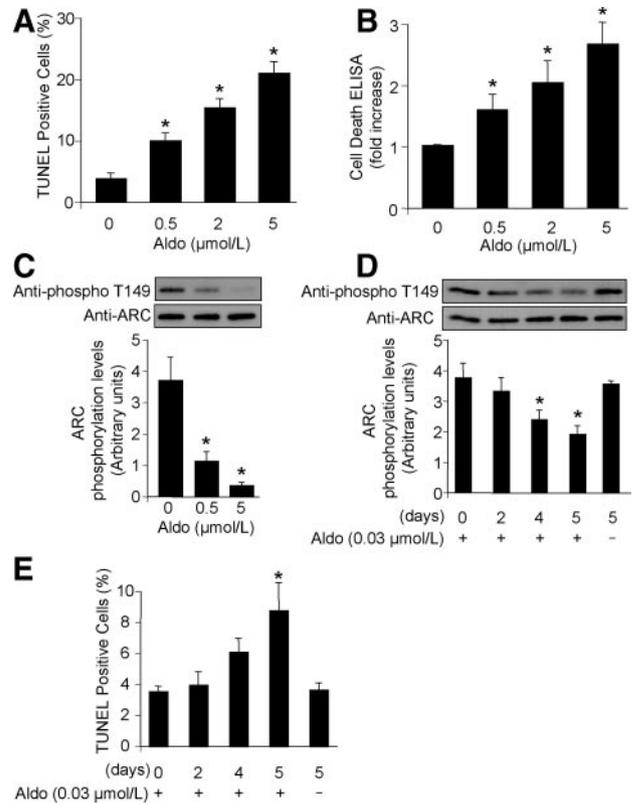


Figure 2. Aldosterone (Aldo) induces a decrease in ARC phosphorylation levels. Aldosterone induces cardiomyocyte apoptosis analyzed by TUNEL assay (A) and cell death ELISA (B). Cardiomyocytes were treated for 48 hours with aldosterone at the indicated concentrations. **P*<0.05 vs control. C, Analysis of ARC phosphorylation levels. Cardiomyocytes were treated for 6 hours with aldosterone. ARC phosphorylation levels were analyzed by immunoblot using the anti-phospho T149 antibody. Top, A representative blot. Bottom, Quantitative analysis of ARC phosphorylation levels by densitometric scanning. **P*<0.05 vs control. D, E, Sustained treatment with aldosterone at a low dose leads to a decrease in ARC phosphorylation levels (D) and an increase in the percentage of apoptotic cells (E). The culture medium containing aldosterone was changed once a day. **P*<0.05 vs day 0. Data in Figure 2 are expressed as the mean±SEM of 3 or 4 independent experiments. All comparisons were performed with Student *t* test.

phorylation levels was related to its dephosphorylation on treatment with isoproterenol or aldosterone.

Calcineurin Is Able to Induce ARC Dephosphorylation In Vitro and In Vivo

It has been documented that isoproterenol and aldosterone can stimulate calcineurin activation and induce apoptosis in a calcineurin-dependent pathway.^{16,19} This led us to consider whether calcineurin was responsible for the reduction in ARC phosphorylation levels on treatment with isoproterenol or aldosterone. We first detected whether calcineurin could directly dephosphorylate the recombinant ARC in vitro. Immunoblot with the anti-phospho T149 antibody revealed that ARC phosphorylation levels were significantly reduced on treatment with the active calcineurin (Figure 3A). We next tested whether calcineurin was responsible for the decrease in ARC phosphorylation levels in vivo. Cyclosporin A or FK506, both inhibitors of calcineurin, could attenuate the

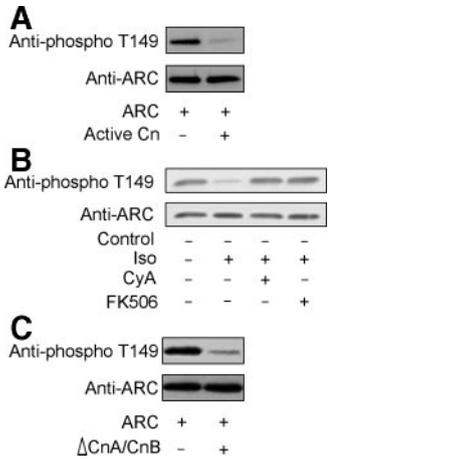


Figure 3. Calcineurin can dephosphorylate ARC in vitro and in vivo. **A**, Calcineurin dephosphorylates ARC in vitro. The purified ARC was incubated with active calcineurin as described in Methods. ARC phosphorylation levels were detected using the anti-phospho T149 antibody. **B**, Cyclosporin A (CyA) and FK506 can inhibit ARC dephosphorylation. Cardiomyocytes were pre-treated for 1 hour with CyA (1 μmol/L) or FK506 (1 μmol/L), treated for 48 hours with isoproterenol (Iso; 50 μmol/L), and then harvested for detection of ARC phosphorylation by immunoblot using the anti-phospho T149 antibody. **C**, The constitutively active calcineurin dephosphorylates ARC. HEK293 cells were transfected with ARC construct or cotransfected with the constructs of ARC and ΔCnA/CnB and harvested 48 hours after transfection. The empty vector was used to maintain the same amounts of plasmids for each transfection. Samples were analyzed by immunoblot with the anti-phospho-T149 antibody.

decrease in ARC phosphorylation levels in cells treated with isoproterenol (Figure 3B). They also could inhibit the decrease in ARC phosphorylation levels induced by aldosterone (data not shown).

We finally tested whether the constitutively active calcineurin could influence ARC phosphorylation levels. The constitutively active form of ΔCnA/CnB²³ was used to coexpress with ARC. To exclude the influence of endogenous ARC, HEK293 cells expressing undetectable endogenous ARC were used for this experiment. Cells transfected with ARC construct showed a high level of phosphorylated ARC. However, cells cotransfected with the constructs of ARC and the constitutively active ΔCnA/CnB showed a low level of phosphorylated ARC (Figure 3C). Thus, it appears that calcineurin is able to induce ARC dephosphorylation.

Phosphorylation Is Required for ARC to Antagonize Isoproterenol- or Aldosterone-Induced Apoptosis

The induction of ARC dephosphorylation by isoproterenol and aldosterone led us to consider whether a link exists between ARC phosphorylation status and isoproterenol- or aldosterone-induced apoptosis. To this end, we used 3 approaches. We first compared the effects of the phosphorylatable wtARC and nonphosphorylatable ARC149A on isoproterenol- or aldosterone-induced apoptosis. WtARC but not ARC149A could inhibit apoptosis induced by isoproterenol (Figure 4A) or aldosterone (Figure 4B). Because treatment with isoproterenol or aldosterone resulted in ARC dephosphorylation as shown in Figures 1 and 2, why could

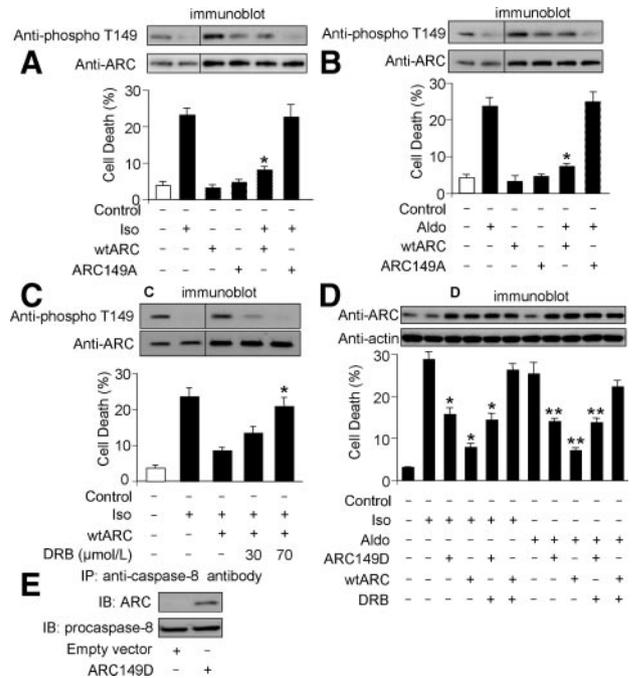


Figure 4. Phosphorylation is required for ARC to inhibit apoptosis induced by isoproterenol (Iso) or aldosterone (Aldo). **A** and **B**, Cardiomyocytes were infected with AdwtARC or AdARC149A at an MOI of 100. Twenty-four hours after infection, they were treated with isoproterenol (50 μmol/L) or aldosterone (5 μmol/L), respectively. Top, ARC phosphorylation levels were analyzed by immunoblot using the anti-phospho T149 antibody. Bottom, Cell death was assessed 48 hours after treatment. **P*<0.01 vs isoproterenol alone or aldosterone alone (Student *t* test). **C**, DRB abolishes the inhibitory effect of wtARC on apoptosis. Cells were infected with AdwtARC at an MOI of 100. DRB was added 6 hours after infection. Twenty-four hours after infection, cells were treated with isoproterenol (50 μmol/L). Top, ARC phosphorylation levels were analyzed by immunoblot using the anti-phospho T149 antibody. Bottom, Cell death was assessed 48 hours after isoproterenol treatment. **P*<0.05 vs isoproterenol plus wtARC (Student *t* test). **D**, ARC149D but not wtARC retains the ability to inhibit apoptosis in the presence of DRB. Cardiomyocytes were infected with AdwtARC (MOI of 100) or AdARC149D (MOI of 100). DRB (70 μmol/L) was added 6 hours after infection. Twenty-four hours after infection, they were treated with isoproterenol (50 μmol/L) or aldosterone (5 μmol/L). Top, The expression levels of wtARC and ARC149D. Bottom, Cell death was assessed 48 hours after treatment. **P*<0.05 vs isoproterenol alone; ***P*<0.05 vs aldosterone alone (ANOVA). **E**, ARC149D can interact with procaspase-8. HEK293 cells were transfected with the constructs of ARC149D or the empty vector pCR3.1. Twenty-four hours after transfection, mitochondria were prepared. Immunoprecipitation (IP) with the anti-caspase-8 antibody was followed by immunoblot (IB) with the anti-ARC antibody. Data are expressed as the mean±SEM of 3 or 4 independent experiments.

enforced expression of exogenous wtARC inhibit isoproterenol- and aldosterone-induced apoptosis? We observed that a significant amount of phosphorylated ARC remained in cells overexpressing wtARC and treated with isoproterenol or aldosterone (Figure 4A and 4B, top). These data indicate that enforced expression of wtARC overwhelms its dephosphorylation induced by isoproterenol or aldosterone.

Second, we tested whether wtARC retained its ability to antagonize isoproterenol-induced apoptosis when its phosphorylation by CK2 was inhibited. DRB is a cell-permeable

CK2 inhibitor^{24,25} that can inhibit CK2 activity, resulting in a reduction in ARC phosphorylation levels.⁴ Thus, DRB was used to inhibit wtARC phosphorylation. The inhibitory effect of wtARC on isoproterenol-induced apoptosis could be abolished by DRB (Figure 4C). DRB itself at concentrations <70 $\mu\text{mol/L}$ could not induce apoptosis as detected by TUNEL assay and cell death ELISA (data not shown).

Third, we constructed an ARC mutant with T149 substituted by an aspartate residue (ARC149D) and tested whether it could functionally mimic phosphorylation. ARC149D could inhibit apoptosis induced by isoproterenol or aldosterone. Strikingly, ARC149D but not wtARC retained the ability to prevent apoptosis in the presence of DRB (Figure 4D). Immunoblot analysis revealed the equal expression of wtARC and ARC149D (Figure 4D, top). We asked whether ARC149D is able to associate with caspase-8. HEK293 cells expressing undetectable ARC were used for the experiment to exclude the influence of endogenous ARC. An interaction between ARC149D and endogenous procaspase-8 could be observed (Figure 4E). Taken together, these data indicate that phosphorylation is required for ARC to regulate apoptosis induced by isoproterenol or aldosterone.

Knockdown of Endogenous ARC Sensitizes Cells to Undergoing Apoptosis on Treatment With Isoproterenol or Aldosterone

To confirm that ARC is indeed related to apoptosis induced by isoproterenol or aldosterone, we tested whether knockdown of endogenous ARC can influence cell susceptibility to undergoing apoptosis on treatment with isoproterenol or aldosterone. ARC-RNAi but not its scrambled form could induce a decrease in ARC levels (Figure 5A). Short-term treatment with isoproterenol (Figure 5B) or aldosterone (Figure 5C) at a low dose could not significantly induce apoptosis in cardiomyocytes. However, at the same concentrations, they could induce apoptosis in cardiomyocytes in which endogenous ARC was knocked down. Thus, it appears that endogenous ARC controls the cell fate on stimulation with isoproterenol or aldosterone.

The Association Between FADD and Procaspase-8 Can Be Inhibited by WtARC but Not ARC149A

In the following experiments, we explored the molecular mechanism by which ARC participates in the regulation of apoptosis induced by isoproterenol or aldosterone. We detected whether ARC could influence calcineurin activity. Calcineurin activity remained at a high level, although wtARC could induce a slight decrease in calcineurin activity (Figure 6A). This suggests that the inhibition of calcineurin itself is not the predominant pathway by which wtARC inhibits isoproterenol- or aldosterone-induced apoptosis.

We further carried out experiments to identify the antiapoptotic pathway of ARC. ARC can inhibit apoptosis by targeting caspase-8.^{4,5} FasL can initiate apoptosis through the caspase-8 pathway.⁴ We thus detected FasL levels on isoproterenol treatment. As shown in Figure 6B, isoproterenol could induce an increase in FasL levels (lane 2). Such an increase in FasL levels could not be affected by wtARC and ARC149A (lanes 3 to 5). We tested whether the upregulated FasL is

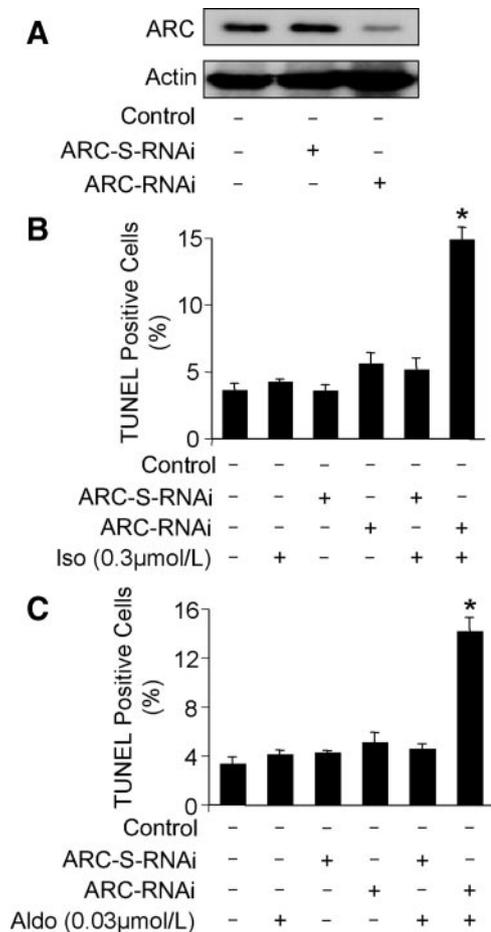


Figure 5. Knockdown of endogenous ARC sensitizes cells to undergoing apoptosis on treatment with isoproterenol or aldosterone (Aldo). A, Cardiomyocytes were infected with the adenoviruses harboring ARC-RNAi or its scrambled form (ARC-S-RNAi) at an MOI of 50. Forty-eight hours after infection, cells were harvested for the detection of ARC levels by immunoblot. B and C, Cardiomyocytes were infected with adenoviruses as described in A and then treated with isoproterenol or aldosterone. The culture medium containing isoproterenol or aldosterone was changed once a day. Two days after treatment, apoptosis was assessed by TUNEL assay. * $P < 0.05$ vs isoproterenol alone or aldosterone alone. Data are expressed as the mean \pm SEM of 3 independent experiments. All comparisons were performed with Student *t* test.

necessary for isoproterenol to induce cell death. Knockdown of FasL could attenuate cell death (Figure 6C). These results suggest that FasL participates in isoproterenol-initiated apoptosis.

We detected whether an association existed between FADD and procaspase-8 in response to stimulation with isoproterenol. As shown in the second panel of Figure 6D, the association between FADD and procaspase-8 could be observed on isoproterenol treatment (lane 2). Enforced expression of wtARC could inhibit the association between FADD and procaspase-8 but not the association between Fas and FADD (lane 3). ARC149A could not interfere with the association between FADD and procaspase-8 (lane 4). The inhibitory effect of wtARC on the association between FADD and procaspase-8 could be abolished by DRB (lane 5). These data indicate that the association between FADD and

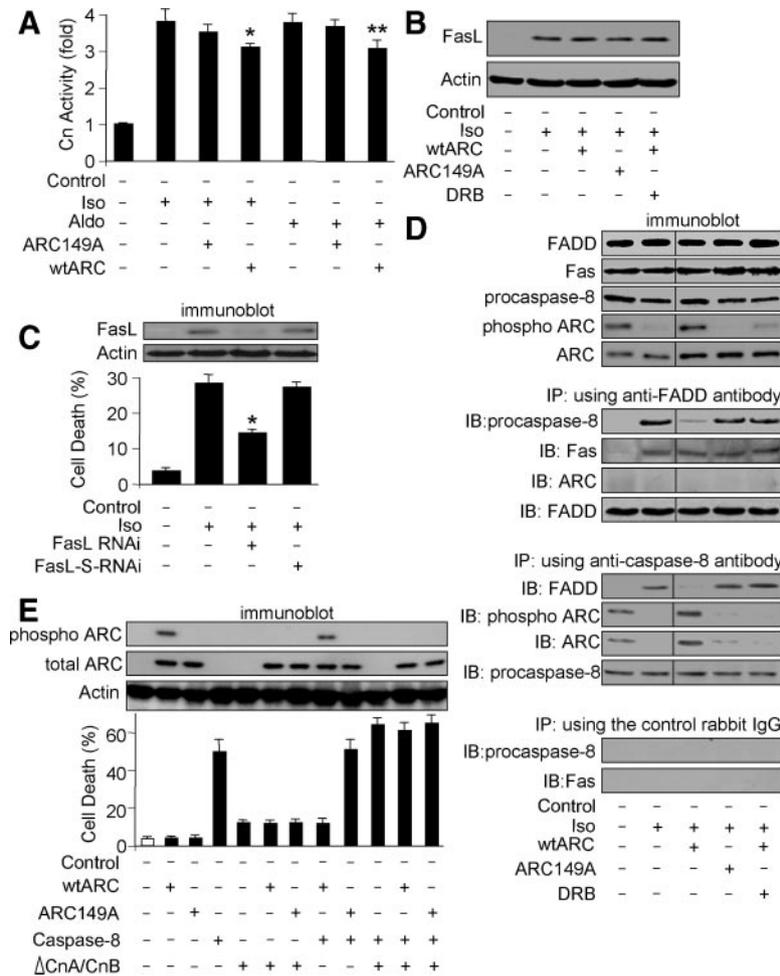


Figure 6. The association between FADD and procaspase-8 can be inhibited by wtARC but not nonphosphorylatable ARC mutant (ARC149A). A, Enforced expression of wtARC can slightly decrease calcineurin activity. Cardiomyocytes were infected with AdwtARC or AdARC149A at an MOI of 100. Twenty-four hours after infection, cells were treated with isoproterenol (Iso; 50 μmol/L) or aldosterone (Aldo; 5 μmol/L). Twenty minutes after treatment, cells were harvested. **P*<0.05 vs isoproterenol alone; ***P*<0.05 vs aldosterone alone (Student *t* test). B, Analysis of FasL expression. Cardiomyocytes were infected with AdwtARC or AdARC149A at an MOI of 100. DRB (70 μmol/L) was added 6 hours after infection. Twenty-four hours after infection, cells were treated with isoproterenol (50 μmol/L). Thirty-six hours after treatment, cells were collected for detection of FasL expression. C, Knockdown of FasL attenuates isoproterenol-induced cell death. Cardiomyocytes were infected with the adenoviruses harboring FasL-RNAi or its scrambled form (FasL-S-RNAi) at an MOI of 80. Twenty-four hours after infection, cells were treated with 50 μmol/L isoproterenol. Top, Thirty-six hours after treatment, cells were collected for detecting FasL expression. Bottom, Cell death was analyzed 48 hours after treatment. **P*<0.05 vs isoproterenol alone. D, Analysis of the associations between FADD and procaspase-8 and the associations between ARC and procaspase-8. Cardiomyocytes were treated as described in B. The levels of FADD, Fas, procaspase-8, the phosphorylated ARC, and ARC were detected by immunoblot (top panel). Immunoprecipitation (IP) was performed with the anti-FADD antibody followed by immunoblot (IB) using the antibodies against procaspase-8, Fas, ARC, or FADD (second panel). Immunoprecipitation was performed with the anti-caspase-8 antibody followed by immunoblot using the antibodies against FADD, phosphorylated ARC, ARC, or procaspase-8 (third panel). Immunoprecipitation with the control rabbit IgG was performed followed by immunoblot with the antibodies against procaspase-8 or Fas (bottom panel). E, ARC dephosphorylation by calcineurin leads to its failure to antagonize caspase-8-induced cell death. HEK293 cells were transfected with the constructs of wtARC, ARC149A, ΔCnA/CnB, or caspase-8. Top, ARC phosphorylation levels were analyzed by immunoblot. Bottom, Cell death was assessed 48 hours after transfection. Data are expressed as the mean±SEM of 3 or 4 independent experiments.

procaspase-8 could be inhibited by wtARC but not ARC149A. We further analyzed whether ARC binds to procaspase-8. As shown in the third panel of Figure 6D, phosphorylated ARC was associated with procaspase-8 (lanes 1 and 3).

Finally, we tested whether ARC dephosphorylation induced by calcineurin can influence its ability to inhibit caspase-8-induced apoptosis. To exclude the influence of endogenous ARC, HEK293 cells expressing undetectable endogenous ARC were used for this experiment. WtARC but not ARC149A was able to inhibit caspase-8-induced apopto-

sis. However, wtARC lost its ability to inhibit caspase-8-induced apoptosis when coexpressed with the constitutively active calcineurin (Figure 6E). Thus, it appears that ARC dephosphorylation by calcineurin leads to the inability of ARC to antagonize caspase-8-induced apoptosis.

Isoproterenol and Aldosterone Require Caspase-8 to Convey Their Apoptotic Signals

We asked whether caspase-8 is a prerequisite for isoproterenol and aldosterone to induce apoptosis. Caspase-8-RNAi but

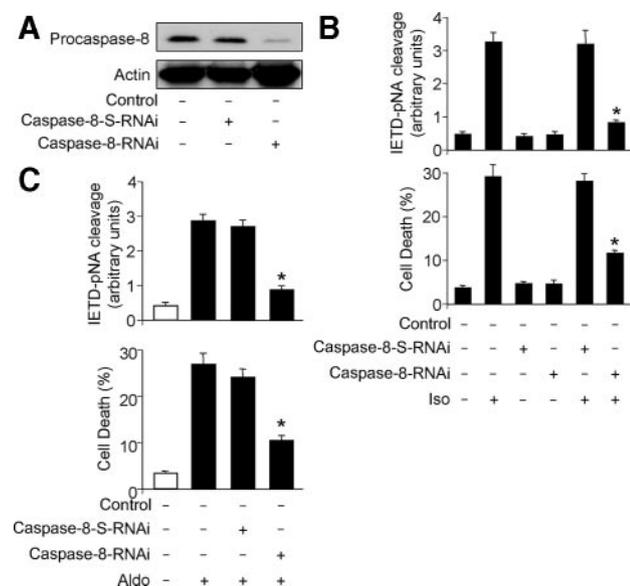


Figure 7. Knockdown of caspase-8 can attenuate cell death induced by isoproterenol (Iso) or aldosterone (Aldo). **A**, Analysis of caspase-8 expression levels. Cardiomyocytes were infected with the adenoviruses harboring caspase-8-RNAi or its scrambled form (caspase-8-S-RNAi) at an MOI of 50. Forty-eight hours after infection, cells were harvested. **B** and **C**, Analysis of caspase-8 activity and cell death. Caspase-8 activity was assayed by the cleavage of its substrate isoleucine-glutamic acid-threonine-aspartic acid conjugated to p-nitroanilide (IETD-pNA). Cells were infected with adenoviruses as described in **A**. Twenty-four hours after infection, cells were treated with 50 $\mu\text{mol/L}$ isoproterenol (**B**) or 5 $\mu\text{mol/L}$ aldosterone (**C**). Cells were harvested for the analysis of caspase-8 activity 36 hours after treatment (top) and cell death 48 hours after treatment (bottom). * $P < 0.05$ vs isoproterenol alone or aldosterone alone. Data are expressed as the mean \pm SEM of 3 independent experiments. All comparisons were performed with Student *t* test.

not its scrambled form could induce a decrease in caspase-8 expression (Figure 7A). Knockdown of caspase-8 could attenuate the increases in caspase-8 activity and cell death induced by isoproterenol (Figure 7B) or aldosterone (Figure 7C). These data suggest that caspase-8 is a necessary component in the apoptotic cascades of isoproterenol and aldosterone.

Discussion

Our present work reveals that calcineurin can induce ARC dephosphorylation in the apoptotic pathways of isoproterenol and aldosterone. ARC dephosphorylation results in its inability to inhibit the association between FADD and procaspase-8 and the consequent apoptosis. Our data reveal a novel apoptotic pathway in which calcineurin dephosphorylates ARC. This finding may help us understand the molecular mechanism by which cardiac apoptosis occurs even in the presence of ARC.

Calcineurin plays a critical role in the coupling of Ca^{2+} signals to cellular responses. In the apoptotic cascades, calcineurin activation can be either proapoptotic or antiapoptotic in cardiomyocytes. Expression of the constitutively active CnA alone prevents apoptosis.²⁶ On the contrary, coexpression of the constitutively active CnA with CnB leads to cardiomyocyte apoptosis.¹⁸ Our present work showed that

calcineurin can dephosphorylate the antiapoptotic protein ARC, resulting in its inability to inhibit apoptosis. Thus, it appears that ARC is a molecular target of calcineurin in its apoptotic cascades.

Although ARC can be dephosphorylated by calcineurin, a significant amount of phosphorylated ARC can be detected in cells overexpressing wtARC and treated with isoproterenol or aldosterone. ARC is phosphorylated by CK2. The levels of CK2 activity are not significantly altered on stimulation with isoproterenol or aldosterone. This may explain why enforced expression of wtARC can overwhelm its dephosphorylation.

ARC can bind to calcium.²⁷ However, our data show that enforced expression of wtARC could slightly reduce calcineurin activity. Calcineurin regulation can be calcium independent.²⁸ One explanation for the limited ability of wtARC to inhibit calcineurin activity is the possible involvement of calcium-independent regulation of calcineurin in the apoptotic pathways of isoproterenol and aldosterone. This hypothesis could be an interesting topic for future study.

The β -adrenergic receptor system is broadly involved in cardiac contraction, growth control, and cell death. Many clinical trials have indicated that β -adrenergic receptor blockers significantly improve survival rates in patients with heart failure by decreasing cardiac remodeling.²⁹ The β -adrenergic receptor agonist isoproterenol promotes cardiac dysfunction resulting from cardiac remodeling, including apoptosis.¹⁴ Aldosterone has recently attracted considerable attention for its involvement in the pathophysiology of heart failure.³⁰ A study performed in patients classified with New York Heart Association class III and IV cardiac failure showed a 30% reduction in morbidity and mortality with the addition of the aldosterone antagonist spironolactone to the conventional therapy.³¹ Eplerenone, a selective aldosterone blocker, similarly shows significant positive effects in patients with acute myocardial infarction complicated by left ventricular dysfunction and heart failure.³² Our present study demonstrates that both isoproterenol and aldosterone can induce ARC dephosphorylation. Consequently, the dephosphorylated ARC is unable to inhibit apoptosis. Thus, inhibition of ARC dephosphorylation could be one molecular approach for the interventional treatment of cardiac disorders related to isoproterenol and aldosterone.

Inhibition of calcineurin cell death signals may include the interferences with calcineurin itself and calcineurin-initiated apoptotic pathways. Our present work reveals that ARC may interfere with the extrinsic apoptotic pathway. Apoptosis can be initiated by the intrinsic pathway that can be targeted by ARC.⁵ It is possible that the intrinsic apoptotic pathway also is involved in calcineurin-induced cell death. For example, aldosterone can induce a decrease in Bad phosphorylation levels,¹⁶ whereas Bad on nonphosphorylation can translocate to mitochondria where it initiates the intrinsic pathway.³³ ARC has recently been reported to be able to interact with Bad.³⁴ The direct or indirect impact of ARC on calcineurin death signals remains to be fully determined.

ARC has previously been shown to undergo ubiquitination and proteasomal degradation in response to oxidative

stress.^{35,36} In contrast, isoproterenol and aldosterone do not significantly decrease ARC protein levels but remove its antiapoptotic ability by causing its dephosphorylation. Thus, differential apoptotic stimuli appear to initiate apoptosis by targeting ARC via distinct mechanisms.

To maintain the heart intact in both structure and function, it is necessary to prevent apoptosis so that the heart does not lose cardiomyocytes. Our finding that ARC loses its antiapoptotic function on dephosphorylation by calcineurin may provide important information for understanding the molecular mechanism by which apoptosis occurs under pathological conditions.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Heart failure is a leading cause of mortality worldwide. The β -adrenergic receptor system is broadly involved in growth control and apoptosis. Many clinical trials have indicated that β -adrenergic receptor blockers significantly improve survival rates in patients with heart failure by decreasing cardiac remodeling. The β -adrenergic receptor agonist isoproterenol promotes cardiac dysfunction resulting from cardiac remodeling, including apoptosis. Aldosterone has recently attracted considerable attention for its involvement in the pathophysiology of heart failure. A study performed in patients with cardiac failure shows a significant reduction in morbidity and mortality with the use of the aldosterone antagonists spironolactone or eplerenone in the therapy. Hitherto, the molecular mechanisms by which isoproterenol or aldosterone induce cardiac apoptosis have not been fully elucidated. Apoptosis repressor with caspase recruitment domain (ARC) is a cardiac-abundant antiapoptotic protein. Its antiapoptotic function is dependent on threonine-149 phosphorylation by protein kinase CK2. The present study reveals that isoproterenol and aldosterone are able to induce ARC dephosphorylation through calcineurin, thereby leading to the inability of ARC to antagonize apoptosis. These data suggest that the dephosphorylation of ARC by calcineurin may constitute an apoptotic pathway in the heart. The present study can provide information for exploring the beneficial effects of this cardiac-abundant protein on apoptosis-related cardiac diseases such as heart failure.