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The Apoptosis Inhibitor ARC Undergoes Ubiquitin-Proteasomal-mediated Degradation in Response to Death Stimuli

*IDENTIFICATION OF A DEGRADATION-RESISTANT MUTANT**

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Efficient induction of apoptosis requires not only the activation of death-promoting proteins but also the inactivation of inhibitors of cell death. ARC (apoptosis repressor with caspase recruitment domain) is an endogenous inhibitor of apoptosis that antagonizes both central apoptosis pathways. Despite its potent inhibition of cell death, cells that express abundant ARC eventually succumb. A possible explanation is that ARC protein levels decrease dramatically in response to death stimuli. The mechanisms that mediate decreases in ARC protein levels during apoptosis and whether these decreases initiate the subsequent cell death are not known. Here we show that endogenous ARC protein levels decrease in response to death stimuli in a variety of cell contexts as well as in a model of myocardial ischemia-reperfusion in intact mice. Decreases in ARC protein levels are not explained by alterations in the abundance of ARC transcripts. Rather, pulse-chase experiments show that decreases in steady state ARC protein levels during apoptosis result from marked destabilization of ARC protein. ARC protein destabilization, in turn, is mediated by the ubiquitin-proteasomal pathway, as mutation of ARC ubiquitin acceptor residues stabilizes ARC protein and preserves its steady state levels during apoptosis. In addition, this degradation-resistant ARC mutant exhibits improved cytoprotection. We conclude that decreases in ARC protein levels in response to death stimuli are mediated by increased ARC protein degradation via the ubiquitin-proteasomal pathway. Moreover, these data demonstrate that decreases in ARC protein levels are a trigger, and not merely a consequence, of the ensuing cell death.

Apoptosis is activated by two major pathways, one involving death receptors (extrinsic pathway) and the other involving the mitochondria/endoplasmic reticulum (intrinsic pathway) (1). Each of these pathways is opposed by several inhibitory proteins, which characteristically act on only one or the other pathway and antagonize very circumscribed step(s) within that pathway. For example, FLIP (Fas-associated death domain protein-like interleukin-1β-converting enzyme inhibitory protein) inhibits the extrinsic pathway by interfering with the activation of procaspase-8 in the Death-inducing Signaling Complex (DISC) (2). In contrast, Bcl-2 (B cell leukemia/lymphoma-2 protein) and XIAP (X-linked inhibitor of apoptosis protein) are examples of intrinsic pathway inhibitors with Bcl-2 blocking permeabilization of the mitochondrial outer membrane via undetermined mechanisms (3) and XIAP inhibiting already activated downstream caspases by blocking substrate access (4). Studies in *Drosophila* and mammalian cells have shown that efficient cell killing requires neutralization of inhibitory proteins as well as activation of death-promoting proteins (5–7).

ARC[3] (apoptosis repressor with caspase recruitment domain) is an endogenous inhibitor of apoptosis that is expressed primarily in terminally differentiated cells, including cardiac and skeletal myocytes and neurons (8, 9). ARC is also induced in a variety of human cancer cell lines and primary human cancers (10, 11). In contrast to most endogenous inhibitors of apoptosis, ARC antagonizes both the extrinsic and intrinsic pathways (12). Inhibition of the extrinsic pathway is mediated by non-homotypic death-fold interactions of ARC with Fas (CD95/Apo-1) and FADD (Fas-associated death domain protein) that preclude the conventional homotypic interactions required for death-inducing signaling complex assembly (12). Inhibition of the intrinsic pathway involves interactions between ARC and the C-terminal regulatory domain of Bax (Bcl-2-associated X protein) that prevent the conformational activation of Bax and its translocation to the mitochondria in response to apoptotic stimuli (12, 13). Overexpression of ARC blocks cell death in response to activators of both extrinsic and intrinsic pathways (9, 12–17). Conversely, knock down of endogenous ARC promotes activation of both pathways (12). Similarly, inactivation of ARC in the mouse increases cardiac myocyte apoptosis in models of myocardial ischemia-reperfusion and hemodynamic overload (18). These

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[3] The abbreviations used are: ARC, apoptosis repressor with caspase recruitment domain; HEK, human embryonic kidney; HA, hemagglutinin.

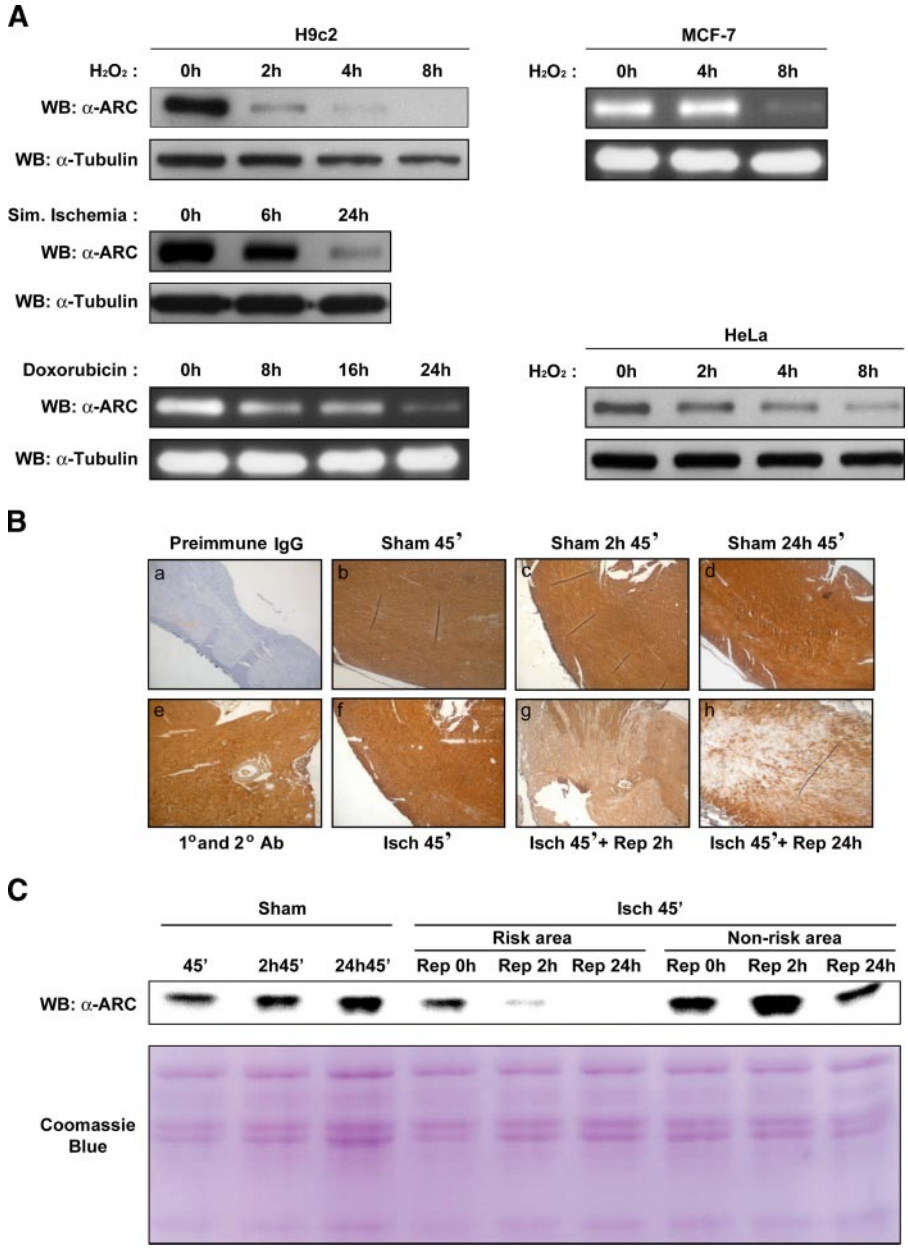

FIGURE 1. **Steady state abundance of ARC protein decreases markedly in response to apoptotic stimuli in cells and intact animals.** *A*, steady state ARC protein levels decrease in various cultured cells in response to different apoptotic stimuli. ARC protein levels were evaluated by immunoblot in H9c2, MCF-7, and HeLa cells following treatment for the indicated times with 400 μM H_2O_2, Simulated Ischemia (defined as omission of serum and glucose from the medium and incubation of cells in an airtight Plexiglas chamber), or doxorubicin (15 μM). In each case, ≥three independent experiments were performed. *B*, ARC protein abundance following myocardial ischemia-reperfusion in intact mice. ARC protein was assessed by immunostaining in mouse heart sections following ischemia (*Isch*) or ischemia-reperfusion (*panels f–h*) or sham operation (*panels b–d*) for the indicated times. *Panel a* is a negative control (preimmune IgG instead of primary antibody). *Panel e* is a positive control (uninstrumented heart stained with primary and secondary antibody). $n = 5$ mice/group. *C*, ARC protein abundance following myocardial ischemia-reperfusion in intact mice. ARC protein levels were assessed by immunoblot following sham operation, ischemia, or ischemia-reperfusion for the times indicated. In ischemia and ischemia-reperfusion groups, ischemic (*risk*) and non-ischemic (*non-risk*) portions of the heart were analyzed separately. $n = 3–6$ mice/group.

observations indicate that ARC is an important inhibitor of apoptosis *in vivo*.

Despite its inhibition of both central apoptosis pathways, even cells that express high base-line levels of ARC can be induced to undergo apoptosis. The susceptibility of ARC-expressing cells to apoptosis has been hypothesized to be due to the fact that ARC protein levels decrease markedly during apoptosis in some systems. These include hypoxia and oxidative stress in the H9c2 muscle cell line and in primary neonatal rat cardiac myocytes, ischemia-reperfusion in isolated, perfused rat hearts, and human heart failure (12, 14, 15, 18–20). The mechanisms responsible for reductions in ARC protein levels during apoptosis and whether these decreases are causally related to the subsequent cell death are not known.

In this study, we show that decreases in ARC protein abundance in response to death stimuli are caused by destabilization of ARC protein. This process is mediated by the ubiquitin-proteasomal pathway. Moreover, mutation of ubiquitin acceptor residues renders ARC resistant to degradation, maintains its steady state levels, and enhances cytoprotection.

EXPERIMENTAL PROCEDURES

Plasmids and Cloning—Mammalian expression vectors for ARC were generated by subcloning the open reading frame of human ARC into pcDNA3.1/pcDNA3.1-HA/pcDNA3.1-Myc-His B/pcDNA3.1-V5-His (Invitrogen). Mammalian expression vectors for procaspase-8 and ubiquitin were obtained from Drs. Gabriel Nunez and Wei Gu, respectively. Mutants derived from pcDNA3.1-ARC-HA were generated using the QuikChange II XL mutagenesis kit (Stratagene) according to the manufacturer's protocol.

Cell Culture and Transfection—H9c2, MCF-7, HeLa, and HEK293 cell lines (ATCC) were grown in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum and 1% penicillin-streptomycin. Primary cultures of neonatal rat cardiac myocytes were prepared as described (21). Cells were transfected with the indicated plasmids using Effectene (Qiagen). H9c2 or HEK293 ARC stable transfectants were generated using Hygromycin (Invitrogen)-resistant vectors.

Antibodies, Immunoblotting, Immunostaining, and Immunoprecipitations—Polyclonal ARC antisera from Cayman or as generated by Dr. M. Crow (14) were used for immunoblotting.

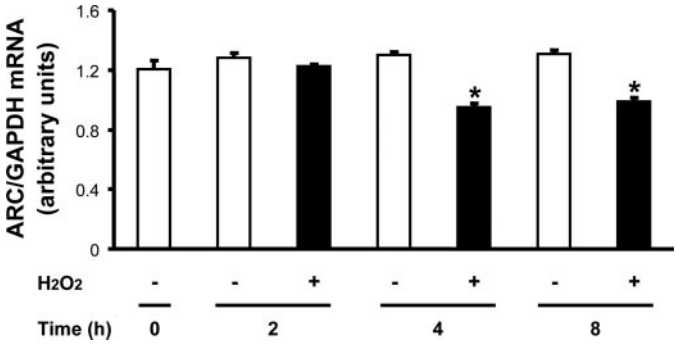

FIGURE 2. **ARC mRNA abundance during apoptosis.** H9c2 cells were not treated or treated with 400 μM H_2O_2 for the times indicated and ARC mRNA levels assessed by quantitative real-time reverse transcriptase PCR. The relative abundance of ARC transcripts were normalized to that of glyceraldehyde-3-phosphate dehydrogenase. *, $p < 0.001$ (4 h) and 0.01 (8 h). Five independent experiments were performed.

The Cayman antibody was also used for immunoprecipitations. A polyclonal ARC antibody (Neomarkers) was used for immunostaining. Additionally, monoclonal antibodies against ubiquitin, HA, Myc (all from Santa Cruz Biotechnology), and α-tubulin (Sigma) were used. Immunostaining was performed on mouse heart sections as previously described (10). Immunoblots and immunoprecipitations were performed as described (12).

Assessment of ARC mRNA Levels—ARC mRNA levels were assessed using quantitative real-time reverse transcriptase PCR. Total RNA was extracted from cell lysates with TRIzol (Invitrogen). First strand cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen). Quantitative real-time reverse transcriptase PCR was performed using the DNA Master Plus SYBR Green I kit (Roche Applied Science) with a LightCycler PCR machine (Roche Applied Science) according to the manufacturer's directions. ARC primers were forward 5′-TGTGCCCAGCAAACAGTG-3′, reverse 5′-CTGGGCATGAAGGGTCATAG-3′. Glyceraldehyde-3-phosphate dehydrogenase primers were forward 5′-TGCCACTCAGAAGACTGTGG-3′ and reverse 5′-GGATGCAGGGATGATGTTCT-3′. The abundance of ARC mRNA was normalized to that of glyceraldehyde-3-phosphate dehydrogenase mRNA.

Assessment of ARC Protein Stability—Cells were washed with phosphate-buffered saline and incubated with methionine/cysteine-free Dulbecco's modified Eagle's medium (ATCC) for 2 h, following which cells were metabolically labeled with 500 μCi of [^{35}S]cysteine (ICN) for 10 h. After labeling, cells were washed with phosphate-buffered saline and chased with complete Dulbecco's modified Eagle's medium containing 10% fetal bovine serum for the indicated times. Lysates were immunoprecipitated for ARC, resolved by SDS-PAGE, and analyzed by autoradiography.

Cell Death Assay—Cell death was assessed by nuclear condensation as described (12).

Mouse Ischemia-Reperfusion Model—Male, 8–10-week-old C57Bl6 mice were subjected to sham operation or ischemia-reperfusion *in vivo* and mouse heart sections and tissue homogenates prepared as previously described (22).

RESULTS

Steady State ARC Protein Levels Decrease in Response to Death Stimuli in Various Systems—Previous work in muscle cells has shown that ARC protein levels decrease in response to oxidative stress and hypoxia (12, 14, 15, 19, 20). To test the generality of this observation, we studied a variety of cell types (including muscle cells and cancer cells that express endogenous ARC) and apoptotic inducers (including oxidative stress, simulated ischemia, doxorubicin, and etoposide). Oxidative stress (hydrogen peroxide) stimulated a brisk and marked decrease in endogenous ARC protein levels in H9c2 cells (skeletal myocyte-like cell line derived from embryonic rat heart), MCF-7 cells (human breast cancer cell line), and HeLa cells (human cervical cancer cell line) (Fig. 1*A*). These decreases in ARC protein abundance were specific as steady state levels of α-tubulin in the same cells remained constant or decreased only modestly. Endogenous ARC protein levels in H9c2 cells also decreased in response to simulated ischemia (serum/glucose/oxygen deprivation) (Fig. 1*A*). Doxorubicin decreased endogenous ARC protein levels in H9c2 cells (Fig. 1*A*), but not in MCF-7 cells (not shown). Etoposide did not affect ARC protein levels in any of these cell types (not shown). Thus, although ARC protein levels are unaffected in some contexts, they decrease in response to oxidative stress in a variety of cell types.

Because oxidative stress is an integral component of myocardial ischemia-reperfusion (23), we postulated that endogenous ARC protein levels would decrease in the hearts of mice subjected to transient left coronary artery ligation. Sham operation or 45 min of ischemia alone, conditions that induce little cardiac myocyte apoptosis (24, 25), had little effect on ARC protein levels (Fig. 1*B*, *panels b–d* and *f*). In contrast, ischemia followed by reperfusion, which elicits significant cardiac myocyte apoptosis (24, 26), induced marked decreases in the abundance of ARC protein (Fig. 1*B*, *panels g* and *h*). Similar results were obtained by Western analysis (Fig. 1*C*). These data demonstrate that endogenous ARC levels in the myocardium decrease dramatically in response to ischemia-reperfusion *in vivo*.

ARC Protein Is Destabilized during Apoptosis—To delineate the mechanism by which apoptotic stimuli decrease ARC protein levels, we first assessed whether ARC mRNA abundance decreases during apoptosis. ARC protein levels decrease most markedly during the first 2 h of hydrogen peroxide-induced apoptosis in H9c2 cells (Fig. 1*A*). Despite this, ARC mRNA levels remained unchanged at 2 h and decreased only modestly at 4–8 h, as assessed by quantitative real-time reverse transcriptase PCR (Fig. 2). Although changes in ARC mRNA levels may play some role in regulating ARC protein levels, these data indicate the kinetics and magnitude of the decreases in ARC protein levels during apoptosis cannot be accounted for by alterations in ARC mRNA abundance.

We next considered whether the stability of ARC protein decreases during apoptosis, an especially attractive hypothesis in light of the rapidity with which ARC protein levels decrease. As measured by pulse-chase, the half-life of endogenous ARC protein was ~16 h in healthy MCF-7 cells (Fig. 3*A*). A similar half-life was observed for transfected human ARC in healthy H9c2 cells (Fig. 3*B*) and HEK293 cells (Fig.

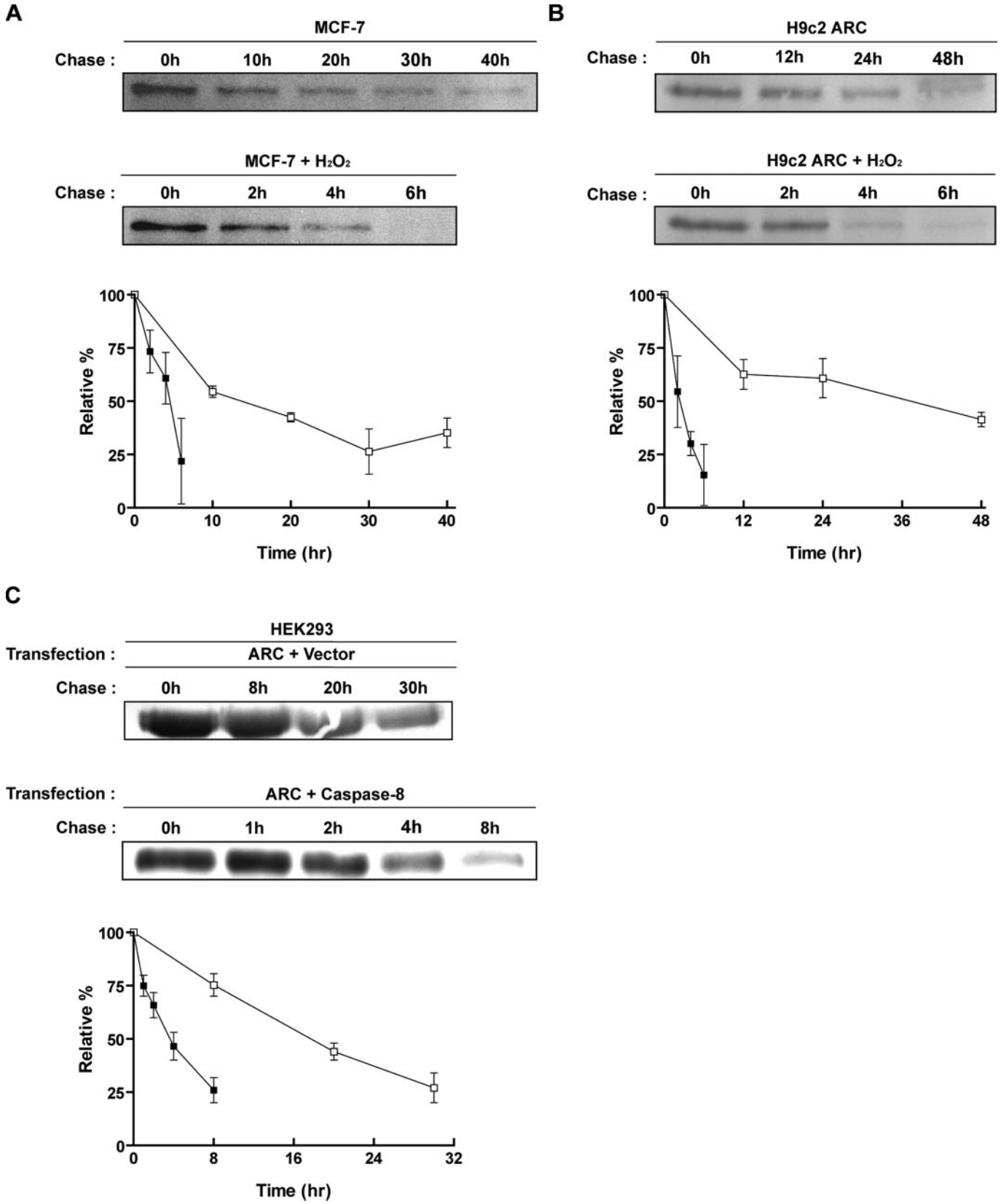

FIGURE 3. **ARC protein half-life decreases markedly during apoptosis.** ARC protein half-life was assessed by pulse-chase. Cells were radiolabeled with [^{35}S]cysteine for 10 h and chased for the indicated times under normal or apoptotic conditions. ARC immunoprecipitates were resolved on SDS-PAGE and autoradiographed. *Graphs* show densitometry (mean ± S.E.) of autoradiographs. Pulse-chase experiments were performed three times. *A*, MCF-7 cells not treated or treated with 400 μM H_2O_2. *B*, H9c2 cells stably transfected with ARC not treated or treated with 400 μM H_2O_2. *C*, HEK293 cells transiently transfected with ARC-HA and either empty vector or procaspase-8 24 h earlier.

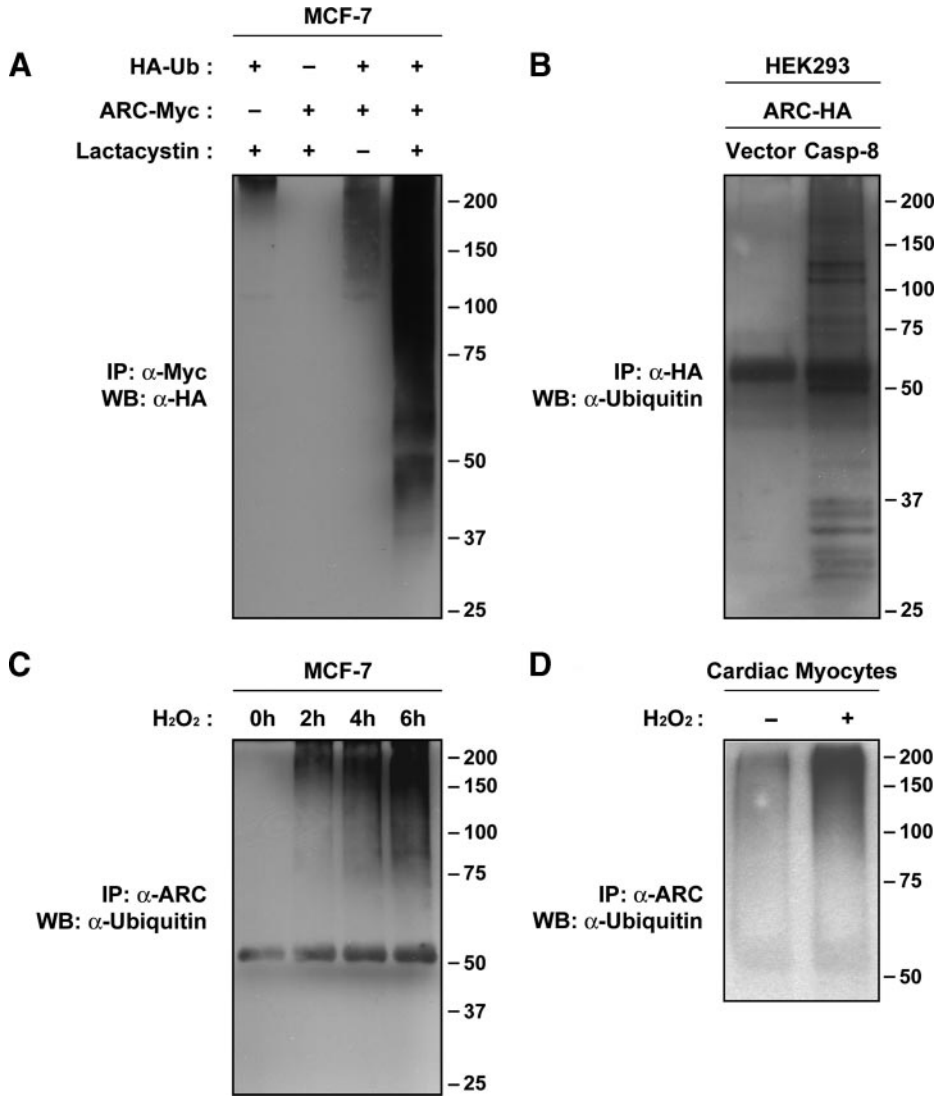

FIGURE 4. **Ubiquitination of ARC during apoptosis.** *A*, ARC can undergo ubiquitination. MCF-7 cells were transfected with HA-ubiquitin (*Ub*) and ARC-Myc constructs in the absence or presence of lactacystin. Cell lysates were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-HA antibody to demonstrate polyubiquitinated ARC. *B–D*, ARC undergoes ubiquitination during apoptosis. *B*, HEK293 cells were transiently transfected with ARC-HA and either empty vector or procaspase-8 (*Casp-8*). Cell lysates were immunoprecipitated with anti-HA, resolved by SDS-PAGE, and immunoblotted with anti-ubiquitin. Similar ubiquitination assays are shown in MCF-7 cells (*C*) and cardiac myocytes (*D*) with and without treatment with 400 μM H_2O_2. Each of these experiments was performed three times.

3*C*). In contrast, treatment of cells with hydrogen peroxide reduced the half-life of ARC protein to ~2–4 h in MCF-7 cells (Fig. 3*A*) and H9c2 cells (Fig. 3*B*). Similarly, activation of apoptosis using procaspase-8 reduced ARC half-life to 4 h in HEK293 cells (Fig. 3*C*). We conclude that ARC protein stability decreases markedly in response to apoptotic stimuli and suggest that increases in ARC protein degradation play an important role in the decreases in steady state ARC protein levels during apoptosis.

ARC Undergoes Polyubiquitination in Response to Apoptotic Stimuli—To test whether the ubiquitin-proteasomal pathway is involved in ARC degradation, we first asked whether ARC could be a substrate for polyubiquitination, which targets proteins for degradation in the proteasome. The co-expression of ubiquitin and ARC in non-apoptotic MCF-7 cells resulted in polyubiquitinated forms of ARC (Fig. 4*A*, *lane 3*), the abundance of which was markedly increased by the 20 S proteasome inhibitor lactacystin (Fig. 4*A*, *lane 4*). These data indicate that ARC can undergo ubiquitination. We next tested whether ARC does undergo ubiquitination in response to apoptotic stimuli. Expression of procaspase-8 in HEK293 cells induced polyubiquitination of co-expressed ARC. Moreover, hydrogen peroxide treatment of MCF-7 cells or primary cultures of neonatal rat cardiac myocytes stimulated polyubiquitination of endogenous ARC. Thus, ARC protein undergoes polyubiquitination in various cell types in response to death stimuli.

The Ubiquitin-Proteasomal Pathway Is Responsible for Decreases in Steady State ARC Protein Levels during Apoptosis—To determine whether the ubiquitin-proteasomal pathway is responsible for the decreased abundance of ARC protein during apoptosis, we created an ARC mutant that is severely resistant to ubiquitination. Ubiquitin moieties are covalently linked to the ϵ-amino groups of lysine residues in the degradation substrate (27). ARC contains lysines at positions 17, 68, and 163. Simultaneous mutation of all three of these lysines to arginines abolished ubiquitination of ARC in response to an apoptotic stimulus (Fig. 5*A*). Pulse-chase studies demonstrated that the triple lysine mutant exhibited markedly increased protein stability under both basal (Fig. 5*B*, *left*) and apoptotic (*right*) conditions. Finally, steady state levels of the triple lysine mutant protein were maintained even 40 h after induction of apoptosis, at which time wild type ARC protein levels were nearly undetectable (Fig. 5*C*). In contrast to the triple lysine mutant, all combinations of single or double mutations of these residues did not significantly alter steady state protein levels during apoptosis (not shown). These genetic data demonstrate unambiguously that decreases in ARC levels during apoptosis are mediated by destabilization of the ARC protein via the ubiquitin-proteasomal pathway.

Degradation-resistant ARC Mutant Confers Improved Cytoprotection—Because the triple lysine ARC mutant protein is resistant to degradation during apoptosis, we tested whether it exhibits improved cytoprotection. Using procaspase-8, apoptosis was induced in HEK293 cell lines stably expressing wild type or triple lysine mutant ARC. Although steady state levels of wild type ARC decreased dramatically during apoptosis, levels

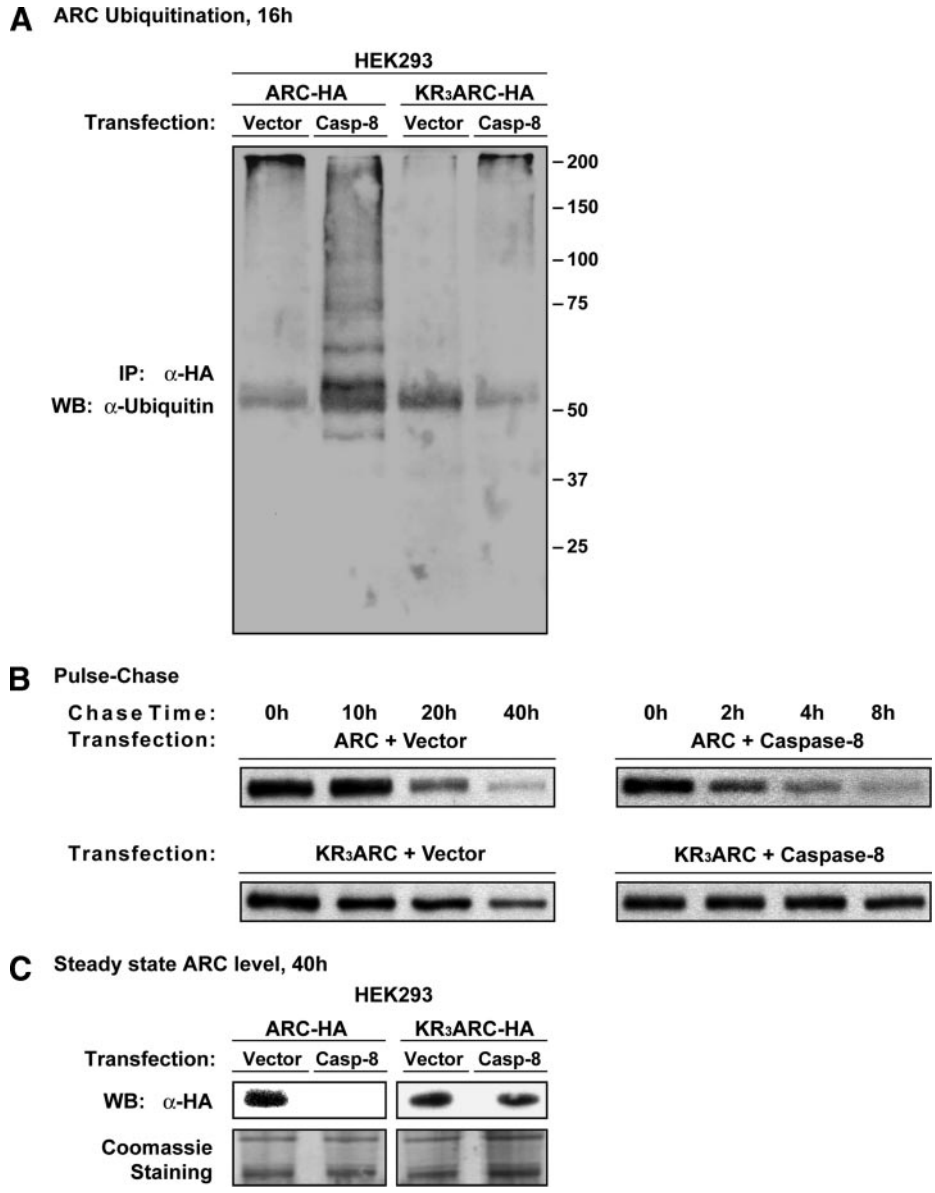

FIGURE 5. **Mutation of potential ubiquitin acceptor sites prevents ARC ubiquitination and degradation and maintains steady state ARC levels.** *A*, HEK293 cells stably expressing ARC-HA or KR_3ARC-HA were transiently transfected with vector or procaspase-8 (*Casp-8*) 16 h earlier. *KR_3ARC* indicates the ARC mutant in which all three lysines are mutated to arginine. Ubiquitination assay was then performed as in Fig. 4. *B*, HEK293 cells stably expressing ARC-HA or KR_3ARC-HA were transiently transfected with vector or procaspase-8 (*Caspase-8*) 16 h earlier. Pulse-chase experiment was then performed as in Fig. 3. *C*, HEK293 cells stably expressing ARC-HA or KR_3ARC-HA were transiently transfected with vector or procaspase-8 (*Casp-8*) and steady state levels of ARC protein assessed 40 h later. These experiments were performed three times.

of triple lysine mutant ARC were maintained (Fig. 6, *upper blot*). Although cell death was decreased by wild type ARC as expected, the triple lysine mutant exhibited significantly more protection (Fig. 6, *graph*). Accompanying this, processing of caspase-8 was decreased more by the triple lysine mutant than by wild type ARC (Fig. 6, *middle blot*). These data show that inhibition of ARC degradation results in enhanced cytoprotection.

DISCUSSION

This study extends the cellular contexts in which ARC protein levels are known to decrease during apoptosis. These decreases are mediated by destabilization of ARC protein via the ubiquitin-proteasomal pathway. Mutation of its ubiquitin acceptor residues renders ARC resistant to degradation during apoptosis, maintains its steady state levels, and enhances its cytoprotection.

Quantitative analysis demonstrated that the marked decrease in steady state ARC protein levels during apoptosis is not explained by changes in ARC mRNA abundance. These data suggest that changes in ARC transcription and mRNA stability do not play major roles in regulating ARC protein levels in this context. These results are consistent with measurements in failing human hearts (18). In contrast, pulse-chase experiments in several cell types demonstrated marked decreases in ARC protein stability in response to different apoptotic stimuli. The magnitude of these changes suggests that increases in ARC protein degradation play a key role in decreases in steady state ARC protein levels during apoptosis.

To determine the mechanism of ARC destabilization during apoptosis, we explored the involvement of the ubiquitin-proteasomal pathway. We found that ARC undergoes polyubiquitination during apoptosis. Moreover, mutation of potential ubiquitin acceptor lysines yielded an ARC mutant that, during apoptosis, failed to be ubiquitinated, was resistant to degradation, and maintained steady state levels. These data provide definitive evidence that the ubiquitin-proteasomal pathway regulates decreases in steady state levels of ARC protein during apoptosis. A related question concerns the identity of the E3 ubiquitin ligase for ARC. As discussed in the accompanying article (28), Mdm2 is one such E3 ligase.

Using stable transfectants with mild overexpression of ARC, we have previously shown that, even in the setting of falling ARC protein levels during apoptosis, physiological levels inhibit cell death (12). These data suggest that maintaining ARC protein abundance at basal levels is sufficient for cell survival. Creation of a degradation-resistant ARC mutant is required to test this hypothesis. The triple lysine ARC mutant maintained ARC protein levels in the face of an apoptotic stimulus, which resulted in significantly more cytoprotection than wild

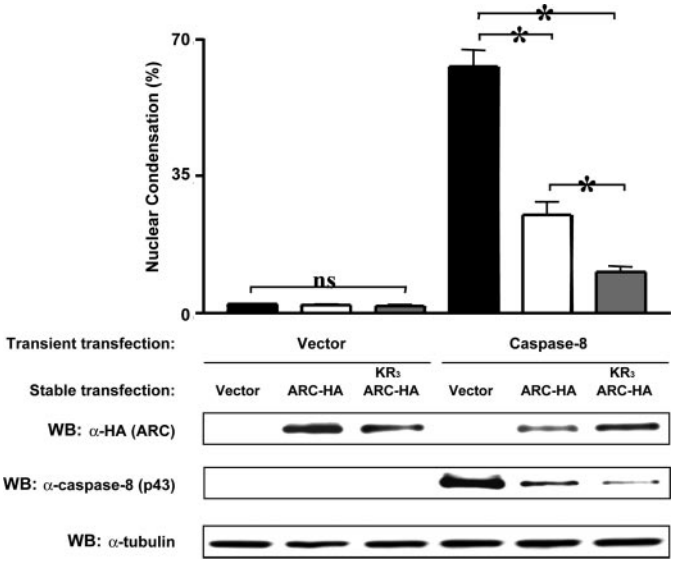

FIGURE 6. **Mutation of ARC ubiquitin acceptor sites enhances cytoprotection.** HEK293 cells stably transfected with vector, ARC-HA, or KR_3ARC-HA were transiently transfected with vector or procaspase-8 (*Caspase-8*) 40 h earlier. Cell death was assessed by nuclear condensation (*graph*). $p < 0.05$ is considered significant (*). Lack of statistical significance is denoted as *ns*. Steady state protein levels of ARC (*top blot*), p43 active caspase-8 (*middle blot*), and α-tubulin (*bottom blot*) were assessed by immunoblotting. Death assay was performed five times. ARC blots were carried out three times. p43 blot was performed once.

type ARC, whose levels decrease during apoptosis. These data provide direct evidence that decreases in ARC protein abundance are indeed an initiating event in apoptosis. Given this causal connection, it will be important to elucidate the pathways that connect death stimuli with ARC degradation. Understanding these pathways may provide therapeutic targets with which to stabilize ARC in heart disease and stroke and destabilize it in cancer.

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