ARC Inhibits Cytochrome c Release From Mitochondria and Protects Against Hypoxia-Induced Apoptosis in Heart-Derived H9c2 Cells

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Abstract—Ischemia induces apoptosis as well as necrosis of cardiac myocytes. We recently reported the cloning of a cDNA that encodes an apoptotic inhibitor, ARC, that is expressed predominantly in cardiac and skeletal muscle. In the present study, we examined the ability of ARC to protect rat embryonic heart–derived H9c2 cells from apoptosis induced by hypoxia, a component of ischemia. We found that H9c2 cells express ARC and that exposure to hypoxia substantially reduces ARC expression while inducing apoptosis. Transfected H9c2 cells in which cytosolic ARC protein levels remain elevated during hypoxia were significantly more resistant to hypoxia-induced apoptosis than parental H9c2 cells or H9c2 cells transfected with a control vector. Loss of endogenous ARC in the cytosol of H9c2 cells was associated with translocation of ARC from the cytosol to intracellular membranes, release of cytochrome c from the mitochondria, activation of caspase-3, poly(ADP-ribose)polymerase (PARP) cleavage, and DNA fragmentation. All of these events were inhibited in H9c2 cells overexpressing ARC when compared with control cells. In contrast, caspase inhibitors prevented PARP cleavage but not cytochrome c release, suggesting that exogenously expressed ARC acts upstream of caspase activation in this model of apoptosis. These results demonstrate that ARC can protect heart myogenic H9c2 cells from hypoxia-induced apoptosis, and that ARC prevents cytochrome c release by acting upstream of caspase activation, perhaps at the mitochondrial level. The full text of this article is available at http://www.circresaha.org. (Circ Res. 1999;85:e70-e77.)

Key Words: apoptosis ■ ARC ■ hypoxia ■ cardiomyocyte ■ cytochrome c

Programmed cell death, or apoptosis, is an evolutionarily conserved process that plays a critical role in embryonic development and adult tissue homeostasis. In humans and mice, dysregulated apoptosis has been implicated in the pathogenesis of cancer and autoimmune, neurodegenerative, and cardiovascular diseases. Apoptosis of cardiac myocytes has recently been recognized as a cellular mechanism of ischemic injury in the heart. Prolonged ischemia appears to result primarily in cardiac myocyte necrosis. If the injury is limited, however, myocyte apoptosis is induced in response to ischemia, as well as during tissue reperfusion in rodents, rabbits, and humans. In addition, apoptosis in cardiac myocytes can be induced by hypoxia alone, serum withdrawal alone, or a combination of hypoxia and glucose and serum deprivation. Myocyte apoptosis has also been observed during cardiac dysfunction associated with mechanical stretch, hodynamic overload, and chronic heart failure.

Recent studies have advanced our understanding of the molecular mechanisms of apoptosis in normal and pathological conditions. Apoptosis is implemented by death machinery whose executionary arm is a family of cysteine proteases termed caspases. These death proteases act in a proteolytic cascade that includes initiator and effector caspases. In mammals, two main caspase cascades have been delineated. The first pathway links caspase-8 to death receptors expressed at the cell surface. In the second pathway, mitochondrial damage induced by diverse extracellular stresses, such as UV irradiation, hyperosmolality, and chemotherapeutic drugs, causes release of cytochrome c from mitochondria into the cytoplasm. In the cytosol, cytochrome c associates with Apaf-1. In the presence of dATP/ATP, Apaf-1 then binds to and activates caspase-9. Activation of initiator caspases-8 or -9 causes proteolytic activation of a common set of downstream proteases, including caspase-3 and -7, to induce cell death. The pathways that initiate apoptosis induced by ischemia and reperfusion in cardiac myocytes are poorly understood. Several factors, including ATP depletion, acidosis, calcium fluxes, and reactive oxygen intermediates have been proposed to cause apoptosis and/or cytochrome c release during ischemia and reperfusion in myocytes.

Several lines of evidence suggest a role for caspases in apoptosis induced by hypoxia and ischemia/reperfusion in heart
cells. First, caspases are activated during apoptosis of cardiomyocytes caused by serum and glucose deprivation, an in vitro experimental condition that mimics aspects of in vivo ischemia. Second, caspase-3, an effector caspase, colocalizes with apoptotic myocytes in an in vivo model of ischemia/reperfusion in the rat. Finally, cardiac myocyte injury triggered by ischemia/reperfusion or hypoxia can be attenuated by a tripeptide inhibitor of caspases. These observations suggest that interfering with caspase activation might provide an effective approach to diminish myocyte injury associated with myocardial ischemia.

Our laboratory has recently identified ARC (apoptosis regulator with caspase recruitment domain), a gene expressed predominantly in cardiac and skeletal muscle that can bind to caspases and reduce apoptosis. However, the ability of ARC to regulate apoptosis in myogenic cells remains to be investigated. In the present study, we show that ARC can suppress hypoxia-induced apoptosis in H9c2 cells, a cell line with myogenic potential derived from embryonic rat hearts. In addition, we provide evidence that ARC regulates apoptosis in part by inhibiting the hypoxia-induced release of cytochrome c from mitochondria in a caspase-independent manner.

Materials and Methods

Cell Culture

The embryonic rat heart–derived myogenic cell line H9c2 (2-1) was obtained from the American Type Culture Collection (Manassas, Va).

Figure 1. Immunoblots of tissue and cell extracts for endogenous ARC expression. Twenty micrograms of total cellular extracts was separated by electrophoresis, blotted to nitrocellulose, and incubated with a polyclonal antibody to rat ARC. A, Extracts from adult heart, adult rat liver, and primary cultures of neonatal rat cardiac myocytes (CM) maintained in the presence or absence of serum. B, Cytosolic extracts (20 μg) from H9c2 cells maintained under normoxic conditions or exposure to 10 hours of hypoxia (2 duplicate cultures). Ponceau staining confirmed equal loading of extracts.

Figure 2. Analysis of ARC-Flag expression in transfected H9c2 clones. A, Fluorescence histograms of H9c2 clones. Cell clones expressing ARC-Flag were analyzed for Flag-epitope expression by flow cytometry. Control represents labeling of pcDNA3-transfected cells (Neo) with anti-Flag monoclonal antibody. B, Immunoblot analysis of H9c2 clones. Cell lysates from H9c2 clones expressing Flag-ARC or control plasmid (Neo) were immunoblotted with an anti-Flag monoclonal antibody. Immunoblot analysis with anti–β-actin monoclonal antibody was used as a loading control. The reaction was developed by enhanced chemiluminescence. Note that the Neo cells do not express Flag-ARC.

DNA Transfection

Culture dishes (100 mm) of subconfluent H9c2 cells were transfected with 10 μg of pcDNA3-Neo or pcDNA3-ARC-Flag using lipofectamine. After 48 hours, the transfected cells were replated in DMEM/10% FCS supplemented with G418 sulfate (500 μg/mL). Colonies derived from single cells were picked and expanded as stably transfected cell lines.

Protein Expression Studies

Endogenous ARC protein expression was detected with an antibody to ARC generated by immunizing rabbits with purified glutathione-S-transferase (GST) rat ARC fusion protein. Expression of epitope-tagged ARC (ARC-Flag) in transfected cells was analyzed by flow cytometry using M2, a mouse monoclonal antibody (mab) against Flag as previously described. Immunoblotting was performed as previously described.
Propidium Iodide Staining

For morphological analysis of apoptosis, cells were fixed, permeabilized, and stained with a solution containing 30% methanol in PBS, pH 7.4, and 20 \( \mu \text{mol/L} \) propidium iodide. Floating and attached cells were harvested, placed on glass slides, and visualized with a fluorescence microscope. The percentage of cells with features of chromatin condensation and nuclear fragmentation was determined and reported as mean \( \pm \text{SEM} \).

Subcellular Fractionation

Fractions enriched in mitochondria and cytosolic proteins (S100) were obtained by differential centrifugation of whole H9c2 cell lysates, as previously described. Equivalent amounts of mitochondrial-rich and cytosolic fractions were subjected to immunoblotting with antibodies specific for Flag, cytochrome c, ARC, or cytochrome oxidase (subunit IV).

Analysis of Caspase-3 Activation

Both floating and attached cells were collected and lysed in isotonic lysis buffer as previously described. Immunoblotting for caspase-3 was performed with polyclonal antibody MF393 (gift of Dr Nancy Thornberry, Merck Research Laboratories, Rahway, NJ). Immunoblotting for poly(ADP-ribose)polymerase (PARP) was performed with mab SA-250 (BIOMOL Research, Plymouth Meeting, Pa) as previously described.

Analysis of DNA Fragmentation

Genomic DNA was isolated from H9c2 cells. Samples were electrophoresed on 1.5% agarose gels to visualize DNA laddering.

Laser Confocal Immunofluorescence Microscopy

Cells (2 to \( 2.5 \times 10^5 \)) were plated on poly-L-lysine–coated cover slides for 24 hours and fixed with cold methanol for 20 minutes at \(-20^\circ\text{C}\). Labeling with anti-Flag antibody was performed as previously described. For identification of mitochondria, MitoTracker Red was added to the cultures, and cells were processed for single and double labeling as previously described.

Statistical Analysis

Data are presented as mean \( \pm \text{SEM} \). The paired analysis between two groups was performed by Student’s \( t \) test.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

ARC Is Expressed in Neonatal and Adult Heart and Primary Cultures of Neonatal Cardiomyocytes

To assess the expression of the ARC protein, a polyclonal antibody was raised in rabbits against a GST-ARC fusion
protein. Figure 1A shows the Western blotting results of extracts from adult rat heart, adult liver, neonatal heart, and primary cultures of neonatal rat cardiomyocytes. The antibody detected a major band in the molecular mass range of \( \approx 33 \) kDa in neonatal and adult heart as well neonatal myocytes cultured in the presence or absence of serum, but as expected from mRNA analyses, nothing in the liver. The antibody also detected a \( \approx 33 \)-kDa band in primary cultures of neonatal cardiomyocytes. The specificity of the antibody was confirmed by showing that when a GST-ARC fusion protein was included during the incubation of the blot with the ARC antibody, the \( \approx 33 \)-kDa band was no longer detected (data not shown). Furthermore, the antibody recognized exogenous ARC expressed from the human ARC cDNA in myogenic H9c2 cells (see Figure 8).

**ARC Overexpression Prevents Hypoxia-Induced Apoptosis**

Cultured myocytes undergo apoptotic cell death after incubation in hypoxic conditions followed by reoxygenation. To study the role of ARC in myogenic cell apoptosis, the cell line H9c2, which is derived from the embryonic rat heart, was exposed to culture conditions that mimicked ischemia. H9c2 cells have previously been used in culture models of myocyte ischemia and reperfusion. As shown in Figure 1B, H9c2 myogenic cells expressed ARC, which decreased in the cytosol of these cells when they were cultured under hypoxic conditions for 10 hours.

To explore the significance of the change in ARC protein expression with respect to apoptosis, stably transfected H9c2 cell lines were isolated that constitutively expressed epitope-tagged human ARC (ARC-Flag). Two clones, termed ARC-5 and ARC-24, showing uniform expression of ARC-Flag, were identified by flow cytometric analyses and selected for further study (Figure 2A). As expected, H9c2 cells transfected with a control plasmid (pcDNA3) lacked ARC-Flag expression. Immunoblotting with an anti-Flag antibody revealed that H9c2 clones ARC-5 and ARC-24, but not cells transfected with pcDNA3 alone (neo), express ARC-Flag (Figure 2B). Comparable cell morphology and proliferation rates were observed in ARC-transfected, pcDNA3-transfected, and parental H9c2 cells (not shown).

Exposure of H9c2 cells to 10 hours of hypoxia along with serum and glucose deprivation followed by 2 hours of reoxygenation resulted in chromatin condensation and nuclear fragmentation in >50% of the cells (64 ± 5% versus 2% in normoxia, “Neo” in Figure 3A and 3B). In contrast to the response of untransfected or control vector–transfected H9c2 cells to hypoxia/reoxygenation, H9c2 clones transfected with ARC displayed very little apoptosis when exposed to the same hypoxic conditions (control, ARC-5, and ARC-24 in Figure 3A and 3B; 64 ± 5% versus 14 ± 4 and 13 ± 3, \( n = 3, P < 0.001 \)). To verify these results, we examined the integrity of genomic DNA in H9c2 cells transfected with ARC or control vector. Both control (“Ctrl”) and ARC-transfected H9c2 cells cultured under normoxic conditions showed no fragmentation of genomic DNA (Figure 3C). In contrast, the DNA from H9c2 cells transfected with a control vector was degraded into nucleosomal fragments when cultured under the conditions of hypoxia/reoxygenation.
Experimental conditions (Figure 5C), indicating that caspases were effectively inhibited under the same concentration of either z-VAD-fmk or z-DEVD-fmk inhibited PARP cleavage, indicating that the results are not due to differential loading of samples. Preincubation of control cells with 100 μmol/L of either z-VAD-fmk or z-DEVD-fmk, two caspase inhibitors, for 30 minutes did not prevent cytochrome c release into the cytosol (Figure 5B). This suggests that inhibition of cytochrome c release by ARC is not caspase dependent. The same concentrations of either z-VAD-fmk or z-DEVD-fmk inhibited PARP cleavage, indicating that caspases were effectively inhibited under the same experimental conditions (Figure 5C).

**ARC Is Distributed in the Cytosol and Mitochondria**

To gain more insight into the mechanism by which ARC inhibits hypoxia-induced apoptosis, we determined the subcellular distribution of exogenous ARC in H9c2 cells by confocal microscopy and immunoblotting of membrane and cytosolic fractions. Laser confocal microscopy revealed that exogenous ARC had a diffuse, granular labeling pattern that was most intense in the perinuclear region of both ARC-5 and ARC-24 H9c2 clones (Figure 6b and 6c). The perinuclear punctate component is typical of proteins that localize to cytoplasmic organelles, most notably in the mitochondria. Labeling with MitoTracker, a rhodamine dye that targets to the mitochondria, showed a punctate pattern similar to that observed with the Flag antibody (Figure 7b). Dual analysis confirmed that some of the punctate pattern of Flag-tagged ARC expression (green fluorescence) colocalized with MitoTracker (red fluorescence) as overlapping red and green pixels was seen as yellow in H9c2 cells expressing ARC-Flag (Figure 7c). To further verify the subcellular distribution of transfected ARC, both cytosolic and membrane/mitochondria-rich fractions were prepared and immunoblotted with anti-ARC and anti-Flag antibodies. The distribution of transfected ARC in the cytosolic and membrane-rich fractions did not change significantly by exposure to hypoxia (Figure 8). In contrast, endogenous ARC expression in normoxic H9c2 cells was detected mostly in the cytosol with much less in the membrane fraction (Figure 8). The distribution of endogenous ARC shifted to the membrane-rich fraction after exposure to hypoxia (Figure 8). The latter result is consistent with the result of Figure 1A showing that exposure of H9c2 cells to hypoxia was associated with a decrease in the cytosolic levels of ARC.

**Discussion**

As demonstrated in this report, hypoxia-induced apoptosis in H9c2 myogenic cells is accompanied by mitochondrial release of cytochrome c, activation of caspase-3, and DNA fragmentation. We found that stable overexpression of ARC, a caspase recruitment domain (CARD)– containing protein expressed primarily in myocytes, protects H9c2 cells from hypoxia-induced apoptosis. In our studies, ARC inhibited the release of cytochrome c from the mitochondria and prevented caspase-3 activation, suggesting that ARC protects cells from hypoxia-induced apoptosis by interfering with noncaspase factors upstream of cytochrome c release.

Many recent studies have indicated that either ischemia or hypoxia alone can trigger cardiomyocyte apoptosis. Although the mechanisms by which hypoxia, ischemia, or ischemia in combination with reoxygenation can trigger cardiomyocyte apoptosis, many mechanisms have been proposed. Some of these factors, such as growth factor withdrawal, UV irradiation, or chemotherapeutic agents, cause apoptosis via a mitochondrial-dependent release of cytochrome c, which induces activation of a complex containing cytochrome c, Apaf-1, and caspase-9, resulting in cleavage and activation of caspase-9. Hypoxia itself may trigger cytochrome c release by inducing reactive oxygen species, increasing cytoplasmic calcium concentration, or decreasing ATP levels, as well as by activating or enhancing levels of proapoptotic proteins, such as p53 or Bax. Hypoxia also enhances levels of hypoxia-inducible factor 1α (HIF-1α), which in turn increases p53 levels. P53 can induce cytochrome c release through its action on Bax, which in turn induces cytochrome c release. It has also...
recently been shown that ischemia can cause cardiac myocyte apoptosis in a p53-independent manner, but the mechanism by which that process occurs has not been ascertained.

In the present study we show, for the first time, that ARC protein levels in the cytosol decrease when H9c2 cells are exposed to hypoxia, with expression appearing to shift to the membrane-rich fraction. Overexpression of transfected ARC maintains cytosolic levels of ARC and inhibits hypoxia-induced apoptosis in this myogenic cell line. We previously had shown that ARC directly interacts with the initiator caspases-2 and -8, blocking apoptosis caused by transfection of caspase-8, Fas, or tumor necrosis factor receptor (TNFR). In addition to this mechanism of inhibition, our present studies identify a separate caspase-independent mechanism by which ARC inhibits cyto-

Figure 6. Transfected ARC exhibits a diffuse and punctate labeling in H9c2 cells. Subcellular localization of ARC by laser confocal microscopy. Immunostaining was performed by anti-Flag monoclonal antibody in Flag-tagged ARC cell lines and pcDNA3-transfected (Neo) H9c2 cells. Images from representative fields are shown. Representative of 3 separate experiments.

Figure 7. ARC colocalizes with MitoTracker in H9c2 cells. Cells were labeled with MitoTracker, a mitochondrial marker dye, and stained with anti-Flag monoclonal antibody to label exogenous ARC. The stained cells were examined by confocal immunofluorescence microscopy. ARC labeling visualized with fluorescein-conjugated goat anti-mouse IgG is shown in green (a and d), and mitochondrial labeling with MitoTracker is shown in red (b and e). Overlapping red and green pixels appear as orange/yellow (c and f). The experiment is representative of 3 separate experiments.
chromosome c release and apoptosis. These results are consistent with the observation in mouse embryo fibroblasts that Apaf-1 and caspase-9, two critical components of the cell death pathway, require cytochrome c for activation, and both are necessary for hypoxia-induced apoptosis. Hypoxia-induced cytochrome c release in our system was insensitive to caspase inhibitors, including the broad-range peptide inhibitor z-VAD-fmk, suggesting that ARC’s inhibition of cytochrome c release in H9c2 cells occurs through a mechanism that did not involve inhibiting caspase activation.

The mechanism by which ARC interferes with cytochrome c release is not understood. It is possible that ARC targets and inhibits proapoptotic proteins that induce cytochrome c release from mitochondria. ARC might also inhibit cytochrome c release from mitochondria directly or indirectly at mitochondrial sites. Several mechanisms have been proposed to explain how cytochrome c is released from mitochondria during apoptosis. The proposed mechanisms include a cytochrome c–specific channel, the opening of the permeability transition pore, and swelling with rupture of the outer mitochondrial membrane. Because ARC is localized in part to the mitochondria, it is conceivable that one of these mechanisms is inhibited directly or indirectly by ARC. For example, ARC may inhibit cytochrome c release through the mitochondrial adenine nucleotide translocator (ANT) and/or the ANT/voltage-dependent anion channel complex (VDAC). This mechanism has also been proposed for Bcl-xL, a prosurvival Bcl-2 family member that also inhibits cytochrome c release from mitochondria. Although ARC lacks a putative anchoring domain that might be involved in insertion of this protein into the outer mitochondrial membrane, it could bind to mitochondria through an adaptor molecule, as it has been proposed for caspase-3. Given that ARC is expressed in heart tissue, an obvious question is whether ARC could protect cardiac myocytes from ischemia and reperfusion injury in vivo. It is important to state that the experiments in the present study were performed in heart-derived H9c2 cells and that it remains unclear whether ARC protects myocytes from hypoxia-induced apoptosis. Additional studies with cardiac myocytes, in culture and in vivo, will be necessary to establish whether ARC interferes with ischemia-induced myocardial cell injury.

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References


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