The Apoptotic Regulatory Protein ARC (Apoptosis Repressor with Caspase Recruitment Domain) Prevents Oxidant Stress-mediated Cell Death by Preserving Mitochondrial Function*

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ARC is an apoptotic regulatory protein expressed almost exclusively in myogenic cells. It contains a caspase recruitment domain (CARD) through which it has been shown to block the activation of some initiator caspases. Because ARC also blocks caspase-independent events associated with apoptosis, such as hypoxia-induced cytochrome c release, we examined its role in cell death triggered by exposure to hydrogen peroxide (H₂O₂) in the myogenic cell line, H9c2. Cell death in this model was caspase-independent and characterized by dose-dependent reduction in ARC expression accompanied by disruption of the mitochondrial membrane potential (Δψₘ) and loss of plasma membrane integrity, typical of necrotic cell death. Ectopic expression of ARC prevented both H₂O₂-induced mitochondrial dysfunction and cell death without affecting the stress kinase response, suggesting that ARCs protective effects were downstream of early signaling events and not due to quenching of H₂O₂. ARC was also effective in blocking H₂O₂-induced loss of membrane integrity and/or disruption of Δψₘ in two human cell lines in which it is not normally expressed. These results demonstrate that, in addition to its ability to block caspase-dependent and -independent events in apoptosis, ARC also prevents necrosis-like cell death via the preservation of mitochondrial function.

One of the principal mechanisms for controlling programmed cell death or apoptosis is through the regulation of protein-protein interactions. These interactions link proximal signals to cellular protease activation and the self-destruction of the cell and are often triggered by ligand-induced conformational changes and/or changes in the relative expression of accessory regulatory molecules. Based on sequence similarities, three prominent interaction motifs involved in apoptosis are recognized. These are the death domain, the death effector domain, and the caspase recruitment domain (CARD)¹ (2–4). Death domains are present on the cytoplasmic domains of many death receptors, such as Fas/CD95 and TNFR1, where they recruit adaptor molecules, such as TRADD and FADD, to the death-inducing signaling complex (1). Adaptor molecules then recruit additional proteins, including initiator caspases which become activated by autoprocessing within the death-inducing signaling complex (5). CARD motifs are found on a majority of initiator caspases (the notable exception being caspase 8) (2), on daptor proteins that interact with and activate these caspases, such as CRADD/RAIDD and Apaf-1 (6, 7), on the IAP family members, cIAP-1 and cIAP-2 (8), and on a growing number of regulatory proteins that modulate intracellular kinase activity and NF-κB activation and influence pro-survival and pro-death intercellular signaling, such as RICK, CARDIAK, and bcl-10/mE10/CIPER (9–13).

ARC (apoptosis repressor with CARD) is a CARD protein that is expressed almost exclusively in myogenic tissue, e.g. the heart and skeletal muscle. ARC selectively interacts with the initiator caspases 2 and 8 and significantly attenuates death receptor-induced apoptosis dependent on the activation of these caspases (14). In the H9c2 rat embryonic heart cell line, ARC expression was shown to decrease in association with activation of hypoxia-induced apoptosis. Ectopic expression of ARC prevented apoptosis, protection being mediated in all or part through the blockade of hypoxia-induced cytochrome c release from the mitochondria (15). Since small peptide broad-spectrum inhibitors of caspases had no effect on this release, these results suggested that, in addition to its potential ability to directly prevent the activation of specific initiator caspases, ARC is also capable of blocking caspase-independent events critical for the execution of apoptosis.

In this study, we have examined the role of ARC in hydrogen peroxide (H₂O₂)-induced cell death in the rat embryonic cell line, H9c2. Cell death mediated by reactive oxygen species is a significant component of ischemia-reperfusion injury in tissues, such as the heart (16, 17). While ischemia causes some cell death on its own, the reintroduction of oxygen during reperfusion is associated with accelerated apoptotic cell death and necrosis, resulting, in part, from a burst of free radical production. H₂O₂ and its reactive by-products are potential mediators of cell death induced by diverse stimuli (18, 19). Under the conditions of the experiments reported in this study, H₂O₂ caused a dose-dependent dissipation of the mitochondrial membrane potential, plummeting intracellular ATP levels, and loss of plasma membrane integrity linked to the early changes in mitochondrial integrity. These changes were accompanied by reduced ARC protein levels and were unaffected by a broad spectrum caspase inhibitor. Moreover, H9c2 cell lines that lacked endogenous ARC expression (H9- cells) were more sen-

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‡ The abbreviations used are: CARD, caspase recruitment domain; ARC, apoptosis repressor with caspase recruitment domain; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; EtD-1, ethidium homodimer-1; MPT, mitochondrial permeability transition; CsA, cyclosporin A.
HeLa cell lines were obtained from ATCC and grown in Dulbecco's modified Earle's medium containing 100 μg/ml penicillin-streptomycin (100 nM MitoTrackerCMXRos was added to live cells for 15 min at 37 °C in the experiment and then averaged over a total of three to six experiments. We also show that ARC provides equivalent protective effects in two nonmyogenic cell lines in which H₂O₂ also causes necrosis-like cell death. These results demonstrate that, in addition to its ability to block caspase-dependent and -independent events in apoptosis, ARC can also prevent cellular changes associated with necrotic cell death through its ability to prevent mitochondrial dysfunction.

EXPERIMENTAL PROCEDURES

Materials—A rabbit polyclonal antibody to ARC was generated as previously described (15). To remove antibodies directed against the N-terminal CARD, which ARC shares with the nucleolar protein, nucleoporins, the antibody was affinity purified using the C-terminal proline-glycine-arginine (P-G-R) rich domain of ARC immobilized on nickel-saturated nitrocellulose (Ni-NTA) agarose matrix (Qiagen). The C-terminal domain of rat ARC was cloned in frame into the bacterial expression vector, pTrcHisTOPO (Invitrogen), to generate a 6XHis-tagged protein. Expression of the protein in bacteria was induced by isopropyl-1-thio-β-D-galactopyranoside and a guanidinium hydrochloride lysate prepared and loaded onto the Ni-NTA column according to the manufacturers instructions (Qiagen). After washing the column to elute nonspecific binding and equilibrating it in 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, the Ni-NTA-agarose matrix with the bound 6XHis-tagged C-terminal domain of rat ARC was incubated with antiiser and washed extensively in equilibration buffer. Antibodies were then eluted in 4 M MgCl₂ (21) and dialyzed overnight in the cold against two changes of phosphate-buffered saline, pH 7.4.

Cell Culture—The H9c2 embryonal rat heart-derived cell line (21) was obtained from the American Type Culture Collection (ATCC) (CRL 1446) and cultured in Dulbecco's modified Earle's medium containing 4500 mg/liter n-glucose and 110 mg/ml pyruvate, supplemented with 10% heat-inactivated fetal calf serum and penicillin (100 units/ml)/streptomycin (100 μg/ml). Cultures were routinely monitored for the presence of ARC and the myogenic determination factor, myoD. A rabbit polyclonal antibody to ARC was generated as described above, and used in Western blotting. The sources of the antibodies used and their dilutions were: ARC (see above, 1:10,000), cytochrome c (Pharmingen 65971A, 1:1000), phospho-specific Erk1/2 (Cell Signaling 9101, 1:1000), phospho-specific p38 (Cell Signaling 9211, 1:1000), total p38 (Santa Cruz, sc-728, 1:1000), total Erk-1 (Santa Cruz, sc-94, 1:5000), and total Erk-2 (Santa Cruz, sc-1647, 1:5000).

RESULTS

H₂O₂ Causes Mitochondrial Membrane Potential (Δψₘ) Dissipation and Loss of Plasma Membrane Integrity in H9c2s—Exposing rat embryonic cardiac H9c2 cells to relatively high doses of H₂O₂ caused disruption of the mitochondrial membrane potential (Δψₘ), and rapid loss of plasma membrane integrity, events characteristic of necrosis. Fig. 1 shows the results of staining untreated and H₂O₂-treated H9c2 cells with markers of Δψₘ (MitoTrackerCMX-Ros), loss of plasma membrane integrity (ethidium dimer-1 (EtD-1)), and cell viability (propidium iodide (calfine-AM)). In untreated cultures, the vast majority of cells were alive, impermeant to EtD-1, trapping calcein-AM within the cell, and displaying an intact Δψₘ (Fig. 1, B, C, and A, respectively). After exposure to 400 μM H₂O₂ for 12 h, however, most cells were dead, showing loss of plasma membrane integrity, as indicated by uptake of EtD-1 and failure to accumulate defective adenovirus encoding human ARC was constructed using the pAdTrack shuttle vector (22). This contains two separate cytomegalovirus promoters to drive either transgene or green fluorescent protein (GFP) expression. Bacterial recombination between the shuttle vector and the replication-defective adenoviral genome, virus amplification, and packaging of virus was performed at the Gene Therapy Center of the University of North Carolina at Chapel Hill. Control virus expressed only GFP. Viral infections were performed on cells in suspension with the optimal amount of virus/cell determined empirically using the GFP marker to achieve 85–90% infection. Infection was performed in serum-free media for 2 h at 37 °C, after which serum-containing media was added and the cells plated on tissue culture plastic. Experimental manipulations of the cells were performed 48 h after infection.

Cytochrome c Release—Cells were seeded at a density of 1 × 10⁶ cells/100-mm dish and then treated 48 h later with 400 μM peroxide in serum-free Dulbecco's modified Earle's medium for 15–60 min. The cells from three dishes were collected for each time point by trypsinization, washed twice in fractionation buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA), and resuspended in 0.15 ml of fractionation buffer supplemented with a protease inhibitor mixture (Sigma P-8340). The cells were homogenized on ice using 100 passes of a tight fitting Dounce homogenizer. Unbroken cells and nuclei were spun down at 2,500 rpm for 10 min. The supernatant was spun at 14,000 rpm for 25 min to separate the “cytotoxic” (supernatant) and “mitochondrial” (pellet) fractions. Cytosolic protein (25 μg) was loaded onto 16% Tris glycine gels (Novex), electroforetically transferred to a polyvinylidene fluoride membrane, and cytochrome c detected by Western blotting as described below.

Western Blotting—ARC and cytochrome c protein levels, caspase activation/cleavage, and mitogen-activated protein kinase activation/phosphorylation were measured by Western blotting. With the exception of the cytochrome c measurements (see above), all extracts were prepared by washing the cells twice in phosphate-buffered saline and then directly scraping them into 1 × SDS sample buffer without reducing agents (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromphenol blue), boiling the extract for 5 min, and then sonicating it to reduce viscosity. Immediately prior to electrophoresis, the sample was reduced by adding dithiothreitol to a final concentration of 50 mM. Samples were heat-denatured and loaded on SDS-polyacrylamide electrophoresis gels for separation. Gels were transferred to polyvinylidene fluoride membranes by semi-dry blotting in 128 mM Tris, 0.96 M glycine, 20% methanol for 2 h at a constant current of 2.5 mA/cm² polyvinylidene fluoride membrane. The membrane was blocked in 5% Amersham Pharmacia Biotech blocking reagent (RP1219) in Western buffer for 1 h at room temperature and incubated overnight in the cold with primary antibody diluted in 2.5% blocking buffer. The membranes were then washed 3 times with continuous agitation in Western buffer (50 mM NaCl, 10 mM Tris, pH 7.2, 1 mM EDTA, 0.1% Tween 20), incubated in 1:10,000 horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech) for 2 h at room temperature, washed again 3 times as above, and then developed in SuperSignal West Pico sub- strate (Pierce Biochemicals) for 3 min. The sources of the antibodies used and their dilutions were: ARC (see above, 1:10,000), cytochrome c (Pharmingen 65971A, 1:1000), cleaved caspase 3 (Cell Signaling D175, 1:1000), phospho-specific Erk1/2 (Cell Signaling 9101, 1:1000), phospho-specific p38 (Cell Signaling 9211, 1:1000), total p38 (Santa Cruz, sc-728, 1:1000), total Erk-1 (Santa Cruz, sc-94, 1:5000), and total Erk-2 (Santa Cruz, sc-1647, 1:5000).
unfixed cells was performed on a Zeiss inverted microscope. Arrows were incubated in EtD-1 and calcein-AM containing media, respectively.

400 H9262 shows the dose response for A, respectively. Fig. 2 /H9004/H9262 increasing concentrations of H2O2 for 12 h. Each mean and loss of plasma membrane integrity were measured in untreated and cells exposed to serum withdrawal alone (no serum), and to M H2O2 and then stained for Δψm dissipation and EtD-1 uptake at 30 min, 1 h, and 2 h after exposure to 400 μM H2O2. Asterisk (*) indicates significantly different from untreated controls, p < 0.001.

undergoing Δψm dissipation and cell death at 400 μM H2O2. Δψm dissipation occurred within the first hour of exposure and was apparent in nearly all of the cells by 2 h. Over this period, cellular ATP levels dropped to <10% (data not shown). The appearance of dead (EtD-1 positive) cells lagged behind the changes in Δψm, suggesting that cell death as measured by EtD-1 uptake may be dependent on mitochondrial dysfunction measured here as a disruption in Δψm. To directly test this, we first pretreated cells with cyclosporin A to determine if the disruption of the Δψm could be blocked by this mitochondrial permeability transition (MPT) inhibitor. Fig. 3A shows that pretreatment with 500 nM cyclosporin A markedly prevented the H2O2-induced Δψm dissipation seen after 2 h. Similar results were seen with bongkrekic acid (data not shown). Fig. 3B shows that CsA not only prevented Δψm dissipation but also the H2O2-induced increase in EtD-1 positive cells, indicating that the loss of plasma membrane integrity was dependent, in large part, on either Δψm dissipation or MPT.

H2O2-induced Cell Death Is Caspase-independent—Fig. 4A shows that H2O2 caused a rapid release of cytochrome c from the mitochondria into the cytosol. While increases in cytosolic cytochrome c can trigger activation of caspase 9 and more distal caspase associated with apoptosis, such as caspase 3, we failed to observe caspase 3 cleavage (a measure of its activation) at any of the H2O2 doses tested (Fig. 4B). Caspase 3 cleavage, however, was readily detectable when cells were incubated with staurosporine and was blocked by preincubating the cells with the broad spectrum inhibitor of caspases, zVAD-fmk, indicating that it was due to processing by upstream caspases. To exclude the possibility that apoptosis may have occurred earlier than any of the times examined or over such an accelerated time frame that it was missed, we pretreated the cells with zVAD-fmk before exposure to H2O2. Fig. 4C shows that this pretreatment had no effect on either Δψm dissipation or cell death. Together these results demonstrate that the necrosis-like cell death we observed with high doses of H2O2 is not a...
down-regulation of the Muscle-specific CARD protein, ARC, Links H2O2 to Cell Death in H9c2s—Because ARC protein levels in H9c2s decrease in parallel with the development of apoptosis caused by hypoxia (12), we examined whether ARC protein levels were also altered in response to H2O2 exposure and, if so, whether these changes were linked to the onset of cell death. Fig. 5A shows a Western blot for ARC expression in various rat tissues and in H9c2s cells. ARC was detected in adult and embryonic heart but not in lung or liver, consistent with previously published Northern blotting data showing that expression of ARC mRNA is restricted to myogenic tissues and the testis (14). The protein detected by our antibody migrates at a slower mobility (between 30 and 36 kDa) than predicted from the primary amino acid sequence of ARC (~25 Kd), due to ARCs proline-rich C-terminal domain. In addition, the protein detected in H9c2 cells (a rat myogenic cell line) migrates faster than the protein in normal rat myogenic tissues. A comparison of the DNA sequence of the ARC cDNAs isolated from H9c2 cells and the rat heart revealed the presence of a 12-amino acid deletion in the proline-rich C-terminal domain of H9c2 ARC. Whether this deletion is of any functional significance is not known, although similar deletions in this region are seen in human and mouse ARCs from both cultured cells and intact tissues. Fig. 5B shows that increasing doses of H2O2 caused dissipation of the mitochondrial membrane potential in H9c2 cells infected with AdTrack.ARC, however, the Δψm remained intact. Fig. 6B shows the combined results of this and several other experiments. Infection with AdTrack.ARC reduced Δψm dissipation from >95% to less than 25% of the cells and the loss of plasma membrane integrity from ~70% to <10% of the cells.

**The Protective Effects of Endogenous ARC**—The experiments described in Fig. 6 showed that enforced expression of ARC could protect cells from the necrotic-like cell death induced by relatively high doses of H2O2. Although endogenous ARC is down-regulated by H2O2, we wished to investigate whether endogenous ARC provided any protection at all from H2O2.

**Summary of the results from three independent experiments (mean ± S.D.). Double asterisk (**) indicates no significant difference between cells receiving no pretreatment and zVAD-fmk.**
do this, we took advantage of a subline of H9c2s that does not express ARC. This ARC-deficient subline was isolated and subcloned in the laboratory as described under "Experimental Procedures" and is referred to as H9—. A Western blot of whole cell extracts from the parental H9c2 and H9— cells confirms that H9— cells do not express ARC (lanes 1 and 2 in Fig. 7A). The bottom panel shows the same extracts probed for Erk1/2 expression to demonstrate that approximately equal amounts of extract were loaded for each sample. Fig. 7B compares the response of H9c2 and H9— cells to increasing doses of H2O2 in terms of EtD-1 staining. The dose-response curve is shifted to the left for H9— cells, indicating that they are more sensitive than the parental H9c2 cells to death-inducing effects of H2O2. In addition, these results show that massive overexpression of ARC seen in the adenovirally infected cells was not necessary to provide a protective effect. In fact, the protection afforded by the H9—/hARC cell lines was greater than that seen following adenoviral delivery of ARC, presumably because of the slight cytotoxicity associated with viral infection of these cells.

One possible mechanism through which ARC could protect cells from oxidative damage is through either the direct quenching of H2O2 or indirect quenching through the increased expression or activation of anti-oxidant defenses. To test this, we examined the effect of enforced ARC expression on H2O2-induced stress kinase activation. In Fig. 8C, the H2O2-induced activation of p38 and Erk1/2 was examined in H9—, parental H9c2, and three of the H9—/hARC cell lines. (No changes in JNK1/2 activation were observed under the conditions of our experiments.) Increased p38 activation as measured by changes in its phosphorylation state was evident in H9— cells and, to a much lesser degree, in one of the H9—/hARC cell lines. In contrast, activation (i.e. phosphorylation) of Erk1/2 in response to H2O2 was seen in all the different cell lines, despite difference in ARC expression. Cells that express ARC tended to have increased basal levels of Erk phosphorylation, but in all cases, peak expression was comparable, occurring between 10 and 15 min after stimulation. These results demonstrate that in ARC-expressing cells, the stress kinase response involving Erk1/2 phosphorylation is unaltered, suggesting that ARC does not directly or indirectly quench H2O2. The activation of p38 in H9— cells may reflect additional changes in gene expression unrelated to its lack of ARC expression or to its increased sensitivity to oxidant stress.

**ARC Prevents H2O2-induced Cell Death in Nonmyogenic Cell Lines**—To determine if the protective effects of ARC against necrosis-like cell death involved in myogenic-specific signaling pathways, we tested whether forced expression of ARC could
attenuate the cytotoxic effects of H2O2 on the MCF-7 and HeLa human cell lines. Fig. 9A shows that increasing doses of H2O2 caused complete Δψm dissipation followed by loss of plasma membrane integrity in MCF-7 cells. These changes were completely prevented by prior infection with the recombinant replication-defective adenovirus expressing human ARC (AdTrack.ARC). Fig. 9B shows that Δψm dissipation also increases with increasing H2O2 levels in another human cell line, HeLa cells. While infection with AdTrack.ARC was not as effective in HeLa as in MCF-7 cells, it still significantly attenuated Δψm dissipation. EtD-1 uptake was not measured in these cells since H2O2 causes the cells to detach from the dish. These results demonstrate that while ARC expression is

mostly confined to myogenic cells it engages pro-survival signaling pathways present in nonmyogenic cell types.

DISCUSSION

We have shown in this study that the muscle-specific repressor of apoptosis, known as ARC, provides an essential link between oxidant stress and cell death in the rat myogenic cell line, H9c2. We showed that H2O2 caused a dose-dependent dissipation of the Δψm and loss of plasma membrane integrity (Figs. 1 and 2) that was accompanied by reductions in ARC protein levels (Fig. 5). We also showed that H9c2 sublines that are ARC deficient (H9−) are more sensitive to the death-inducing effects of H2O2 (Fig. 7). Enforced expression of ARC by either adenoviral-mediated gene transfer (Fig. 6) or the creation of stably integrated cell lines expressing ARC (Fig. 9) dramatically increased the resistance of parental H9c2 and H9− cells to H2O2. Although adenoviral gene transfer resulted in large overexpression of the protein, it is especially noteworthy that expression of the transfected ARC transgene in the same. ARC is a protein composed principally of two domains: a C-terminal domain consisting of multiple proline/glutamate repeats and an N-terminal CARD. CARD proteins have been shown to block cytochrome c release and hence, the downstream caspases activated by the cytochrome c-dATP-Apaf-1-caspase-9 complex. Notably, this inhibition was mediated by a caspase-independent effect of ARC (15). Most other CARD
proteins potentiate apoptosis, including RAID/CRADD (6), bcl-10 (9–12), Apaf-1 (7), and CARD4/Nod1 (23, 24), although the inhibitors of apoptosis, cIAP-1 and -2, contain a CARD (8). The CARD domains of cIAP-1 and -2, however, are not required for their direct interaction with caspases and the blocking of apoptosis (25), but may function to disrupt other CARD-dependent apoptotic events (26). ARCs ability to block not only the activation of initiator caspases 2 and 8 and to modulate caspase-independent mitochondrial events associated with caspase-dependent apoptosis and caspase-independent cell death distinguishes it from the IAPs.

The early loss in both plasma membrane integrity and cellular ATP levels we observed in this study are hallmarks of necrotic cell death. Because loss of plasma membrane integrity can also occur secondary to apoptosis, inhibitors of apoptosis, such as ARC, could prevent the secondary appearance of the necrosis-like phenotype by preventing primary apoptosis. This scenario, however, is inconsistent with the time course of plasma membrane disruption and the almost complete loss of cellular ATP, as well as with the fact that no evidence for caspase activation was obtained after H2O2 treatment and that broad spectrum caspase inhibitors had no effect on loss of plasma membrane integrity or dissipation of the $\Delta \psi_m$ in response to H2O2 (Figs. 4 and 6).

Understanding the mechanism(s) through which H2O2 causes cell death is likely to provide insight into the mechanism of ARCs protective effects. Our data indicate that H2O2-induced cell death in H9c2 cells is likely to start with disruption of the mitochondria, possibly caused by the MPT. In this study, we measured the dissipation of the $\Delta \psi_m$ as an indicator of mitochondrial dysfunction. $\Delta \psi_m$ dissipation is often used as a direct marker of MPT, but not all changes in $\Delta \psi_m$ are caused by MPT. To investigate the potential role of the MPT, we pretreated cells with cyclosporin A (CsA), an inhibitor of cyclophilin D, which is an inner membrane protein and a component of the mitochondrial permeability pore complex (27, 28). (Similar results were seen in cells pretreated with bongkrekic acid, a ligand for another member of the mitochondrial permeability, the adenine nucleotide translocator.) The fact that CsA prevented both H2O2-induced $\Delta \psi_m$ dissipation and loss of plasma membrane integrity (Fig. 3) indicates that 1) $\Delta \psi_m$ dissipation is secondary to MPT, and that 2) events triggered by either MPT and/or $\Delta \psi_m$ dissipation cause cell death. H2O2 induces ability to induce MPT may be due to a direct oxidation of thiol groups on the pore complex (29, 30) or on calcium transport systems on the mitochondria, the endoplasmic reticulum, or the plasma membrane (31, 32).

Based on our results, the likely sequence of events occurring during H2O2-induced cell death is that H2O2 activates the MPT, which in turn leads to cytochrome c release, disruption of the $\Delta \psi_m$ and the uncoupling of oxidative phosphorylation, the likely cause for plummeting cellular ATP levels. In the absence of ATP, cytochrome c fails to activate caspase 9 since Apaf-1 requires ATP as a cofactor (7) and there is, therefore, no apoptotic component to the process of cell death. Instead, the loss of ATP likely contributes to the rapid loss of plasma membrane integrity and cell death via a necrosis-like mechanism. The fact that enforced expression of ARC also prevents H2O2-induced $\Delta \psi_m$ dissipation (Figs. 6, 9, and 10) as well as cytochrome c release suggests that ARC blocks cell death by either stabilizing the $\Delta \psi_m$ in the presence of the MPT or directly preventing the MPT. That mitochondria may be an important target for ARCs preventative actions is consistent with previous studies showing that ARC translocates from the cytosol to the mitochondrial membrane fraction in response to an apoptotic stimulus (hypoxia) and prevents the release of cytochrome c (15), an event which is not caspase-dependent and not predictable based on the original description of ARC as an inhibitor of selective initiator caspases.

ARCs ability to block both apoptotic and necrosis-like cells is reminiscent in some respects of that of the anti-apoptotic members of the bcl-2 family. Thus, enforced bcl-2 expression completely blocks both staurosporine-induced apoptotic and necrotic cell death in Jurkat cells (33), while other investigators have shown that bcl-2 retards necrotic cell death in PC12 caused by respiratory chain poisons such cyanide, rotenone, or antimycin A and partially protects Rat-1 fibroblasts from high dose hydrogen peroxide (34, 35). Given the importance of the mitochondria as gatekeepers for both necrosis and apoptosis (28, 36, 37), the shared ability of bcl-2 and ARC to block both apoptotic and necrotic-like cell death may be linked to their constitutive or transient localization to the mitochondrial membrane fraction.

In summary, we have presented data demonstrating that ARC links oxidative stress to cell death in H9c2 cells. H2O2 causes necrotic-like cell death that is caspase-independent, triggered by disruption of mitochondrial function, and accompanied by down-regulation of ARC expression. Enforced expression of ARC prevents cell death by preserving mitochondrial function. While ARC expression is, for the most part, limited to myogenic cells and tissues, it engages pro-survival signaling pathways common to nonmyogenic cells as well. These results identify a novel function for the CARD protein, ARC, and suggest that it may be particularly effective in counteracting both apoptotic and necrotic-like forms of cell death to provide protection against cellular damage accumulated during ischemia and in response to the reperfusion of ischemic heart tissue.

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