Journal of Cellular Biochemistry 104:818–825 (2008)

Expression and Modification of ARC (Apoptosis Repressor With a CARD Domain) is Distinctly Regulated by Oxidative Stress in Cancer Cells

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Abstract Apoptosis repressor with a CARD domain (ARC), which has been shown to protect against oxidative stress-induced apoptosis, was initially found to be highly expressed in terminally differentiated tissues like heart and skeletal muscle. Recently, we and others have found that ARC is also expressed at high levels in multiple cancer tissues and cell lines. Here, we compared the regulation of ARC in response to oxidative stress between cancer cells and other types of cells. Similar to cardiomyocyte cell line H9c2 cells, cancer cells with reduced ARC expression were significantly more sensitive to oxidative stress. However, oxidative stress did not down-regulate ARC expression in cancer cells as it did in H9c2 cells. We further found that in H9c2 cells oxidative stress regulates ARC protein expression post-translationally through proteasome-mediated degradation. In cancer cell line HeLa, the majority of ARC exists in phosphorylated state in the absence of oxidative stress, whereas in H9c2 cells only marginal amount of ARC was phosphorylated under similar conditions. Our data suggest that the high level of ARC protein and the constitutive phosphorylation of ARC in cancer cells may play an important role in the protection of cancer cells against oxidative stress. J. Cell. Biochem. 104: 818–825, 2008.

Key words: ARC; oxidative stress; cancer; apoptosis

Evasion of apoptosis is a hallmark of tumorigenesis [Hanahan and Weinberg, 2000]. It has been demonstrated that a variety of cancer cells have defects in the apoptotic machinery, either in those elements involved in the execution of apoptosis or those engaged in the regulation of apoptosis. Two ways cancer cells evade apoptosis are via the elevation of the level of anti-apoptotic factors and/or suppression of pro-apoptotic factors. A typical example is the up-regulation of the anti-apoptotic protein Bcl-2 in B-cell lymphoma [Cleary et al., 1986]. It is also well known that the proapoptotic protein Bax is dysfunctional due to mutations in several cancer cells [Rampino et al., 1997; Saitoh et al., 1999]. Thus, in general, there is an intimate linkage between tumorigenesis and apoptosis. Therefore, it is not surprising that several emerging therapeutic targets involving apoptosis are being pursued in cancer treatment [LaCasse et al., 1998; Holcik et al., 2001; Zaffaroni et al., 2005]. One of the therapeutic targets is the inhibitors of apoptosis protein (IAP) family of proteins, which inhibit caspase activities, mainly the activity/activation of caspase −3, −7, and −9.

A new IAP-like apoptosis inhibitor, apoptosis repressor with a CARD domain (ARC), has been identified [Koseki et al., 1998]. Some of the basic functions of ARC have been defined in the rat cardiomyocyte cell line H9c2 [Koseki et al., 1998; Ekhterae et al., 1999; Neuss et al., 2001; Li et al., 2002; Ekhterae et al., 2003; Gustafsson et al., 2004]. ARC was found to be highly expressed in heart and skeletal muscle with little or no detectable expression in other tissues. It has two domains of which the CARD domain appears to be involved in homotypic

Grant sponsor: NIH grant; Grant number: AG20210 (B. H.); Grant sponsor: NIA training grant; Grant number: AG021890 (Y.-Q. Z.).

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Received 27 September 2007; Accepted 19 November 2007

DOI 10.1002/jcb.21666

protein–protein interaction. ARC is translocated to mitochondria during apoptosis and phosphorylation at threonine 149 is required for this translocation. ARC protects the integrity of mitochondria and prevents the mitochondrial localization of Bax. Recently, ARC was reported to be over-expressed in several cancer cell lines [Mercier et al., 2005; Wang et al., 2005]. We hypothesize that elevation of ARC gene expression may contribute to the resistance of these cancer cells to apoptosis.

In the present study, we found that ARC expression is elevated in all cancer cell lines examined and reduction of ARC expression by siRNA in one cancer cell, HeLa, significantly lowered its resistance to oxidative stress. At the same time, we found that majority of ARC was constitutively phosphorylated in cancer cells but not in H9c2 cells. Furthermore, oxidative stress did not alter the level of ARC expression in two cancer cell lines as it did in H9c2 cells. Finally, the level of ARC expression appears to be post-translationally regulated by a proteasomal-mediated pathway in H9c2 cells. These findings suggest that the level and regulation of ARC expression as well as its modification is different between cancer cells and other cell types.

MATERIALS AND METHODS

Cell Culture and Medium

HeLa cells (ATCC) were maintained in Dulbecco's modified eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cell lines which were kindly provided by Dr. Bert Vogelstein from Johns Hopkins University, were maintained in RPMI medium supplemented with 10% FBS at 37°C with 5% CO_2. Mouse embryonic fibroblasts (MEFs) were isolated from pregnant mice at 12.5–14 days post-copulation. MEFs were maintained in DMEM supplemented with 10% FBS at 37°C with 5% CO_2. H9c2 was treated with 200 μM H_2O_2 and cancer cells were treated with 400–800 μM H_2O_2 for 12–24 h to induce apoptosis [Zhang and Herman, 2006].

Plasmid Construction and Transfection

Human ARC was subcloned into pcDNA3.1 vector (Invitrogen) and $pECFP_{N1}$ (Clontech) by PCR. Threonine 149 of ARC was mutated to alanine using the above vectors as templates by site-directed mutagenesis according to the manufacturer's instructions (Stratagene). Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In transient transfection experiments, H9c2 cells were transfected and incubated for 24 h before treatment. For stable transfection, stable cell clones were generated by selection with 1 mg/ml of G418 or 300 μg/ml of hygromycin for at least 2 weeks after initial transfection and maintained with antibiotics throughout the culturing period. Protein expression was confirmed by fluorescence microscopy and Western blot analysis.

The Design and Transfection of siRNA

ARC or p53-specific siRNA oligos were synthesized by Qiagen. The coding sequences of siRNA oligos are: ARC 5′-CCCAGTACCGCTGGAAGTGAA-3′; p53 5′-CACAGCGACAGGGTCACCTAA-3′. siRNA oligos were transfected into cells using Oligofectamine reagent following manufacturer's instructions (Invitrogen). Briefly, cells were plated in medium without antibiotics the day before transfection so that a 30–50% confluence was achieved at the time of transfection. siRNA oligos (100 nM) was used for each transfection. Protein expression was examined using Western blot analysis 60 h after initial transfection. Treatment was performed at 60–70 h after transfection.

Western Blot and Detection of Phosphorylation

The protein concentration of cell lysates was determined by BCA protein assay reagent (Pierce). Equal amount of protein was loaded onto 12% tris-glycine SDS–PAGE gel and separated at 100 V for 1 h. The protein was transferred to PVDF membrane and blocked with 5% milk in TBST (Tris-buffered saline with Tween-20) buffer. ARC was detected with anti-ARC antibody (Oncogene). Cytochrome *c* was detected with anti-Cytochrome *c* antibody (BD Sciences). Anti-β-actin antibody (Sigma) was used to indicate the loading of proteins.

The phosphorylation of ARC was detected by immunoblotting of immunoprecipitated samples from cell lysate using anti-phosphothreonine antibody cocktail (CalBiochem). Briefly, H9c2 cells over-expressing ARC-CFP or T149A-CFP mutant or HeLa cells transfected with ARC were treated with 200 or 800 μM H_2O_2 respectively for 12 h. Cells were lysed using RIPA buffer (1% NP-40, 1% sodium

deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH7.2, 1% Trasylol, protease inhibitor cocktail from Roche). Supernatant was immunoprecipitated by agarose-conjugated anti-GFP antibody according to the manufacturer's instructions (Promega). The immunoprecipitated samples were separated by SDS–PAGE gel and detected with anti-phosphothreonine antibodies.

Statistical Analysis

Data are expressed as the mean ± SEM. The significance of differences between samples was analyzed by one-way or two-way ANOVA. For each time point or experiment, triplicate or quadruplicate samples were analyzed. A P-value of <0.05 was considered statistically significant.

RESULTS

ARC Expression in Multiple Cancer Cell Lines and its Response to Oxidative Stress

It has been reported that ARC expression increases in cancer cells [Mercier et al., 2005; Wang et al., 2005]. We examined the level of ARC expression in multiple cancer-derived or transformed cell lines using Western blotting analysis. The expression of ARC was elevated in most of the cancer cell lines tested except Jurkat cells, and also elevated in some transformed cell lines such as 293T (Fig. 1A). The level of ARC expression observed in cancer cells is comparable to or greater than that of H9c2 cells. Interestingly, there is only very low level of ARC in MEFs. The size of ARC band is higher in cells from neuronal lineage, possibly due to post-translational modifications. The human ARC in cells other than neuronal lineage examined in this study is smaller than rat and mice due to short gene coding sequence.

ARC has been shown to be down-regulated by hypoxia and oxidative stress in the cardiomyocyte cell line H9c2 [Neuss et al., 2001]. When ectopically expressed ARC was introduced into H9c2 cells, resistance to ROS was increased [Ekhterae et al., 1999]. In this study, we compared the regulation of ARC expression in cancer cell lines versus other types of cells. ARC expression is down-regulated in H9c2 cells in a time-dependent manner when challenged with 200 μM H_2O_2 (Fig. 1B). However, there was no change in ARC expression in HeLa cells when treated with 800 μM H_2O_2 during 24 h period (Fig. 1B). This same finding was also observed in another tumor cell line HCT116 (Fig. 2A). This suggests that the regulation of ARC in cancer cells is different from that in H9c2 cells.

The Regulation of ARC Expression by Oxidative Stress

To address the question of how oxidative stress regulates ARC gene expression in H9c2 cells and cancer cells, we first tested the regulation mechanism at the transcriptional level. One important transcription factor, p53, has been demonstrated to either up-regulate proapoptotic or down-regulate anti-apoptotic genes [Moroni et al., 2001]. To see the effect of p53 on ARC gene expression, we blocked the expression of p53 with gene-specific siRNA oligos (Fig. 2A, left panel). When treated with 200 μM H_2O_2 for 4–24 h, the lack of p53 did not affect the course of ARC gene expression in H9c2 cells (Fig. 2A, right panel), suggesting that ARC is not transcriptionally regulated through p53 in response to oxidative stress. We also treated p53 isogenic MEFs and HCT116 colon cancer cell lines with either 400 μM (MEFs) or 800 μM (HCT116) H_2O_2 for various time periods. There were no observable changes in ARC protein expression during the course of treatment with H_2O_2, regardless of the background of p53 (Fig. 2B), further suggesting that p53 is not essential in regulating ARC gene expression.

To further exclude the possibility of transcriptional regulation of ARC in response to oxidative stress, ARC mRNA level in either HeLa cells or H9c2 cells was examined using quantitative RT-PCR with specific primers for either human or rat ARC. The results did not show difference in ARC mRNA levels before and after oxidative stress in either HeLa cells or H9c2 cells (Fig. 2C), indicating that ARC expression is not regulated at the transcriptional level by oxidative stress, further confirming the results obtained using p53 isogenic cells. Next, we examined the possibility of a post-translational regulation of ARC protein in response to oxidative stress in H9c2 cells. As shown in Figure 2D, addition of the proteasomal inhibitors lactacystin at 50 μM or MG132 at 25 μM prevented the decrease in ARC protein level in H9c2 cells when treated with 200 μM H_2O_2. This data indicate that the proteasome-mediated degradation of ARC is a major

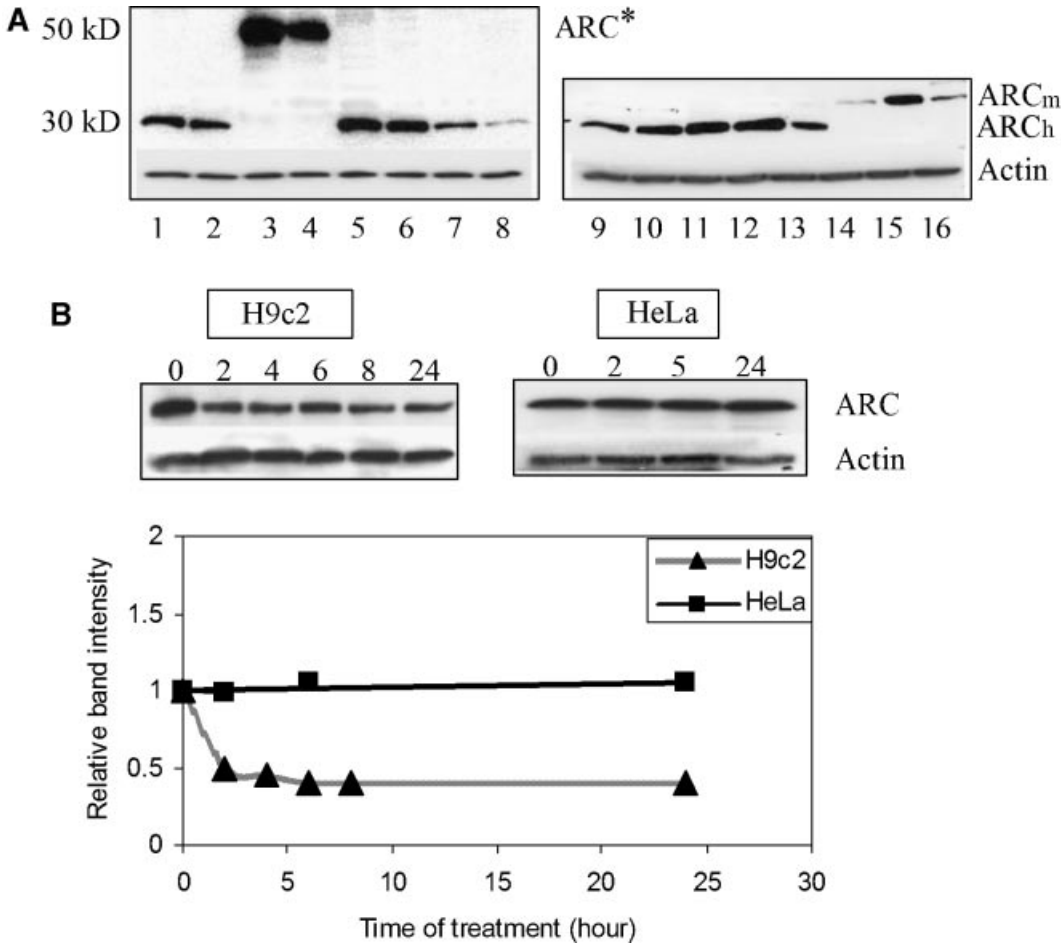

Fig. 1. Expression of ARC in cancer cell lines. **A**: ARC expression in multiple cancer cells lines at normal culture conditions. Lane 1—MDA435 (human breast cancer), lane 2—MDA231 (human breast cancer), lane 3—CATH.a (mouse tumorigenic neuronal cell line), lane 4—PC12 (rat neuroblastoma precursor cell), lane 5—HeLa (human cervical carcinoma), lane 6—HepG (human hepatocarcinoma), lane 7—RD (human cardiomyoblast), lane 8—Jurkat (human T lymphoma), lane 9—HCT116 $p53^{+/+}$, lane 10—HCT116 $p53^{-/-}$ (human colon cancer), lane 11—H417 (human prostate cancer), lane 12—L_NCap (human small lung cancer), lane 13—293T (transformed human fibroblast), lane 14—AML12 (transformed mouse hepatocytes), lane 15—H9c2 (rat cardiomyocyte), lane 16—MEFs. ARC was blotted with anti-ARC antibody (Leinco) at 1:1,000. Actin was detected with anti-β-actin specific antibody at 1:4,000 (Sigma). ARCm, mouse ARC; ARCh, human ARC; ARC*, neuronal ARC. **B**: ARC expression in response to oxidative stress. H9c2 or HeLa cells were treated with H_2O_2 (200 and 800 μM respectively) for the time specified on the graph. Cells were harvested at each time point and the cell lysate was subjected to Western blot analysis after separated by SDS–PAGE gel as described in Materials and Methods. The band intensity was analyzed by densitometry and normalized to the intensity of actin.

mechanism for the decrease in ARC protein expression in H9c2 cells in response to oxidative stress.

Phosphorylation of ARC in Cancer Cells

ARC was not reduced by oxidative stress in some cancer cells tested earlier in this study. This may be due to post-translational modifications that prevent its rapid degradation. Phosphorylation of ARC at threonine 149 is required for its anti-apoptotic function, suggesting that phosphorylated ARC might be less prone to degradation. To test this hypothesis, we further compared the phosphorylation of ARC among different types of cells during oxidative stress. Same amount of total cell lysate was immunoprecipitated with either anti-GFP or anti-ARC antibody and detected with phosphor-protein specific antibodies. As indicated in Figure 3A, H9c2 cells exhibited a very low level of ARC phosphorylation before the addition of H_2O_2. In contrast, the majority of ARC in HeLa cells was phosphorylated prior to H_2O_2 treatment (Fig. 3B). Treatment with hydrogen peroxide increased the amount of phosphorylated ARC in H9c2 cells significantly, but did not change the

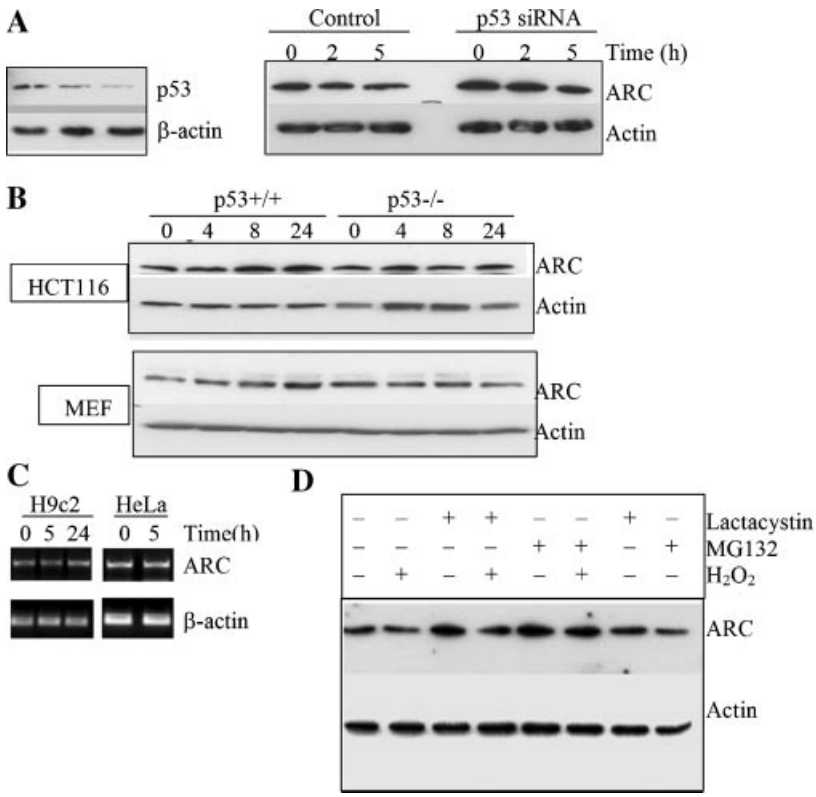

Fig. 2. The regulation of ARC gene expression in response to oxidative stress. **A**: Effect of p53 on the expression of ARC in H9c2 cells in response to oxidative stress. H9c2 cells were transfected with p53-specific siRNA oligos and control oligos. **Left panel**: Expression of p53 protein was detected with p53-specific antibody. IR, irrelevant siRNA; #1 and #2 are two clones of H9c2 cells transfected with p53-specific siRNA oligos. **Right panel**: H9c2 cells were treated with 200 μM of H_2O_2 for 0, 2, and 5 h respectively and immunoblotting of ARC and beta-actin was performed on whole cell lysate. Control–H9c2 cells with control siRNA; p53siRNA–H9c2 cells treated with p53-specific siRNA #2. **B**: Colon cancer cell lines HCT116 isogenic for p53 locus (wild type and homozygous deletion) and p53 isogenic MEFs ($p53^{+/+}$ and $p53^{-/-}$) were treated with 800 μM H_2O_2 for various time periods before harvested. Cell lysate from different time points was subjected to Western blot analysis as described in Materials and Methods. **C**: Semi-quantitative RT-PCR to detect mRNA level in H92 cells and HeLa cells in response to oxidative stress. H9c2 cells were treated with 200 μM H_2O_2 for 0, 5, and 24 h. HeLa cells were treated with 400 μM H_2O_2 for 0 and 5 h. **D**: ARC protein level in H9c2 cells were modulated by proteasomal-mediated degradation. H9c2 cells were treated with 200 μM H_2O_2 for 5 h in the presence or absence of either 50 μM lactacystin or 25 μM MG132.

amount of phosphorylated ARC in HeLa cells significantly, evidenced by the similar intensity of phosphor-protein bands in HeLa cells, but a clear difference in H9c2 cells. Mutation in threonine 149 eliminated the oxidative stress-induced phosphorylation in H9c2 cells, indicating threonine 149 is the major phosphorylation site in ARC during oxidative stress (Fig. 3A).

Reduction of ARC Expression by siRNA Decreased the Resistance of HeLa Cells to Oxidative Stress

To further test the role of ARC in oxidative stress in cancer cells, we employed the siRNA technique to reduce the expression of ARC in one cancer cell line, HeLa cells, using ARC-specific siRNA oligos. ARC protein expression was successfully reduced by siRNA oligos in HeLa cells (Fig. 4A lane 2 and 3). HeLa cells with or without reduced ARC expression were challenged with various ROS inducers for 20 h at the specified concentrations. Cell death was compared at the end of treatment. As shown in Figure 4B, reduction of ARC expression in HeLa cells significantly increased cell sensitivity to oxidative stress, compared to cells with control siRNA only (Fig. 4B). The differences of cell death between control HeLa cells and ARC-siRNA transfected HeLa cells were observed with hydrogen peroxide (25–30% vs. 55–65%), t-butyl hydroperoxide (7–25% vs. 45–55%), rotenone (25% vs. 50%), and antimycin A (35% vs. 55%), suggesting that the increased expression of ARC in HeLa cells contributes to their resistance to oxidative stress.

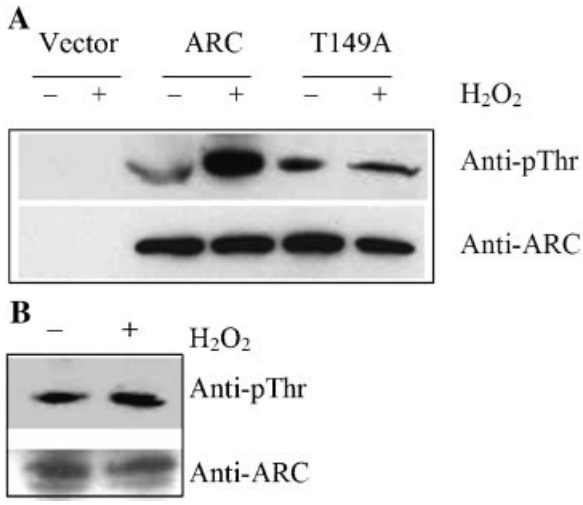

Fig. 3. Phosphorylation of ARC during oxidative stress. **A**: Stable H9c2 cell clones with vector (CFP), ARC, or ARC-T149A mutant were treated with 200 μM H_2O_2 for 6 h. **B**: HeLa cells were treated with 800 μM H_2O_2 for 6 h. At the end of treatment, equal amount of whole cell lysate was immunoprecipitated with anti-ARC (HeLa cells: **panel B**) or anti-CFP antibody (H9c2 cells: **panel A**) followed by separation with SDS–PAGE gel for Western blot analysis as described in Materials and Methods. Phosphorylated ARC was detected with anti-phosphothreonine antibodies (see Materials and Methods). The same sample was also blotted by anti-ARC antibody in a separate membrane. p-Thr: Phosphorylated ARC.

DISCUSSION

Cancer cells have elevated intrinsic resistance to a variety of apoptotic stimuli including oxidative stress and chemotherapy [Pelicano et al., 2004]. Either mutation of apoptotic genes or alteration of apoptotic gene expression contributes partially to this phenomenon [Miller and Samid, 1995; Guchelaar et al., 1997; Li et al., 2001; Real et al., 2002; Pelicano et al., 2004; Zaffaroni et al., 2005]. A variety of factors involved in the apoptotic process have been shown to be either up- or down-regulated in various types of cancers, including Bcl-2 [Tsujimoto et al., 1985], survivin [LaCasse et al., 1998; Lu et al., 1998], and p53 [Sharpless and DePinho, 2002]. A new type of caspase inhibitor, ARC has recently been identified as an inhibitor for oxidative stress-induced apoptosis [Ekhterae et al., 1999; Neuss et al., 2001; Zhang and Herman, 2006] and has been found to be elevated in breast cancer cells and other terminally differentiated cells [Mercier et al., 2005; Wang et al., 2005]. The goal of this study is to examine the role of ARC in oxidative stress response in cancer cells.

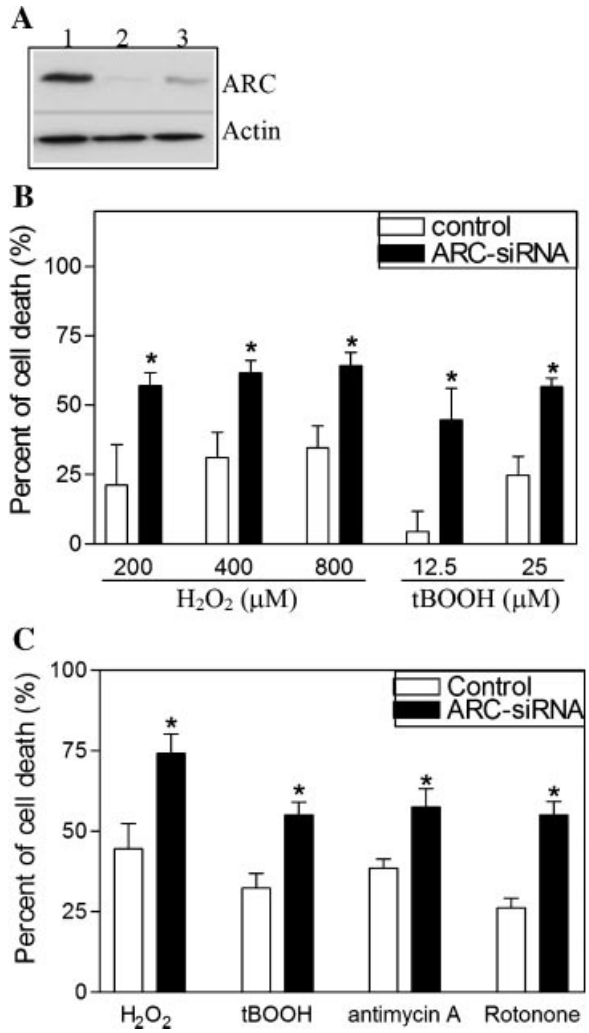

Fig. 4. Reduction of ARC in HeLa cells increases the sensitivity of HeLa to oxidative stress. **A**: ARC-specific siRNA reduced ARC expression significantly in HeLa cells. Lane #1: HeLa cells with control siRNA, lane #2 and #3: HeLa cells with ARC-specific siRNAs. **B**: HeLa cells with ARC-specific siRNA (#2) became more sensitive to different concentrations of H_2O_2 or tBOOH. Transiently transfected HeLa cells with control siRNA or ARC-specific siRNA were treated with different ROS inducers for 20 h. Cell viability was analyzed by the MTT assay as described in Materials and Methods. **C**: HeLa cells with reduced ARC are more sensitive to a variety of ROS inducers. H_2O_2: 800 μM, tBOOH—t-butyl hydroperoxide: 25 μM, antimycin A: 25 μg/ml, rotenone: 10 μM. Data were statistically different at $P < 0.05$ (*) between control and siRNA transfected HeLa cells.

In this study, we revealed for the first time that ARC may be potentially an oncogenic factor. We found that ARC expression is elevated in a wide range of cancer cell lines from different tissues including breast, prostate, lung, liver, and colon cancer. The high level of ARC protein expression is not affected by

oxidative stress in cancer cells, as is in non-tumorigenic H9c2 cells. The majority of ARC protein is phosphorylated in HeLa cells under normal cultural conditions, whereas H9c2 cells have very low level of phosphorylation under similar conditions. Reduction of ARC expression by siRNA significantly increased the sensitivity of HeLa cells to oxidative stress, indicating that ARC is an important factor contributing to the resistance of cancer cells to apoptosis.

The finding that reduction of ARC in cancer cells increased their sensitivity to oxidative stress places ARC as a potential therapeutic target for cancer treatment. However, studies employing other types of cancer cells are necessary to confirm this phenomenon. In addition, more studies are needed to quantify the percentage of ARC being phosphorylated in cancer cells under different conditions and how this modification affects the proteasomal degradation of ARC. Our data suggest that the phosphorylated form of ARC may be more resistant to proteasome-mediated degradation and contributes to the elevated resistance of cancer cells to oxidative stress. In H9c2 cells, majority of ARC is not phosphorylated under normal cultural conditions, but can be phosphorylated when placed under oxidative stress. However, the phosphorylation rate may be not fast enough to catch up with the proteasomal-mediated degradation process, therefore leads to the decrease of ARC in H9c2 cells and ultimately apoptosis. Since majority of ARC is phosphorylated in cancer cells and phosphorylated ARC behaves differently from unphosphorylated ARC, further studies should be conducted to characterize which form of ARC, phosphorylated or unphosphorylated, is the best therapeutic target. Therefore, a separate approach to reduce the protection of cancer cells may involve the inhibition of phosphorylation of ARC.

It is also necessary to examine the role of ARC in the resistance to other types of apoptotic stimulation, including radiation and chemical therapy, which have broad clinical implications in cancer treatment.

In summary, this study demonstrated that the high level of ARC expression in cancer cells contributed significantly to their intrinsic resistance to oxidative stress-induced apoptosis. Oxidative stress failed to down-regulate the level of ARC protein in these cells. In addition, cancer cells maintained constitutively a high level of phosphorylated ARC protein, which is critical to the anti-apoptotic function of ARC. The constitutive high level of ARC expression and high level of phosphorylated ARC indicated that ARC might be an important oncogenic factor. The high level of ARC protein in cancer cells might also contribute to their resistance to other apoptotic stimuli. However, further studies are required to compare apoptosis under different conditions using different approaches including the siRNA approach. Because the reduction of ARC gene expression in cancer cells enhanced apoptosis of these cells, it appears that ARC might be an important therapeutic target for cancer treatment.

ACKNOWLEDGMENTS

This work was supported by NIH grant AG20210 to B. H. Y.-Q. Z. is a recipient of NIA training grant AG021890 directed by Dr. Olivia M. Pereira-Smith at the University of Texas Health Science Center at San Antonio.

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