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Down-regulation of aldose reductase renders J774A.1 cells more susceptible to acrolein- or hydrogen peroxide-induced cell death

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Abstract

Aldose reductase (AR) is abundantly expressed in a variety of cell lineages and has been implicated in the cellular response against oxidative stress. However, the exact functional role of AR against oxidative stress remains relatively unclear. This study investigated the role of AR in acrolein- or hydrogen peroxide-induced apoptosis using the J774.A.1 macrophage cell line. Ablation of AR with a small interference RNA or inhibition of AR activity significantly enhanced the acrolein- or hydrogen peroxide-induced generation of reactive oxygen species and aldehydes, leading to increased apoptotic cell death. Blockade of AR activity in J774A.1 cells markedly augmented the acrolein- or hydrogen peroxide-induced translocation of Bax to mitochondria along with reduced Bcl-2 and increased release of cytochrome c from the mitochodria. Taken together, these findings indicate that AR plays an important role in the cellular response against oxidative stress, by sequestering the reactive molecules generated in cells exposed to toxic substances.

Keywords: *Aldose reductase (AR), acrolein, hydrogen peroxide, oxidative stress, reactive oxygen species (ROS).*

Introduction

Oxidative stress has been implicated in various pathological and physiological conditions, such as cardiovascular disease, cancer, neurodegenerative disorders and ageing [1–4]. Oxidative stress is characterized by the production of a diverse group of reactive species, known collectively as reactive oxygen species (ROS), which are generated by a number of pathways. ROS induce the lipid peroxidation that propagates and amplifies ROS-mediated cellular damages. The byproducts of lipid peroxidation, such as the α,β-unsaturated aldehydes that include 4-hydroxy-2,3-*trans*-nonenal (4-HNE) and acrolein (2-propenal), mediate many of the detrimental effects of oxidative stress [5,6].

Among the unsaturated aldehydes, acrolein is the most reactive electrophilic aldehyde. It shows high reactivity toward nucleophiles in amino acids, peptides, proteins and DNA, forming covalently-modified adducts [5,7,8] and thereby causing cell death [9]. Acrolein is widely distributed in living environments due to the photochemical reaction or the

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incomplete combustion of organic materials such as wood, leaves, fuels or tobacco [5]. In addition to environmental resources, acrolein is also produced intracellularly during lipid peroxidation [7] and during the metabolic conversion of the anti-tumour drug cyclophosphamide [10]. Acrolein is therefore a widely present compound that is hazardous to human health if not properly metabolized.

Aldose reductase (AR; EC 1.1.1.21), a member of the NADPH-dependent aldo-keto reductase family, has been implicated in the cellular response to toxic aldehydes and oxidative stress in several cell lineages [11–16]. With its broad substrate specificity, AR catalyses not only methylglyoxal, a toxic aldehyde product of glucose [17], but also a variety of reactive aldehydes, such as acrolein [18] and 4-HNE [19] that are produced during lipid peroxidation. In this respect, AR is a detoxifying enzyme that increases cellular resistance to toxic injury. Notably, the enzyme is transcriptionally regulated by ROS and its own substrates, methylglyoxal and 4-HNE [11,15,16,20]. Induction of AR expression may represent an adaptive response to increase cellular resistance to toxic injury. Indeed, our recent study indicates that the antioxidant curcumin exhibits anti-apoptotic properties by up-regulating AR expression [12].

Macrophages, through their generation of reactive molecules, play pivotal roles in inflammatory and host defense mechanisms against foreign invaders [21]. However, ROS generated by activated macrophages in large amounts can cause unwanted damage to neighbouring cells and to the ROS-producing macrophages themselves. A population of RAW264.7 macrophages, when exposed to repeated challenges with non-lethal doses of lipopolysaccharide and interferon-γ to elicit nitric oxide (NO) generation show resistance to the toxic effects of NO through increased expression of heat shock protein 70 [22]. In this context, we investigated the role of AR in oxidative stress-induced cell damage by regulating AR expression and activity. We report here the key role of AR in protecting cells from oxidative stress-induced damage and apoptotic cell death, by sequestering the toxic molecules generated in J774A.1 macrophages.

Materials and methods

Cell culture and reagents

The J774A.1 murine macrophage cell line was maintained in RPMI-1640, containing 100 U/ml penicillin and 100 μg/ml streptomycin and supplemented with 10% heat-inactivated foetal bovine serum, at 37°C under an atmosphere of 95% air and 5% CO_2.

Gene silencing with a small interfering RNA (siRNA) and treatment of cells

Using the RNAiFect transfection reagent (Qiagen, Valencia, CA), J774A.1 cells were transfected with 100 nM control siRNA (Ambion, Austin, TX) or siRNA designed against nucleotides (5′-AATCGGTGTCTC CAACTTCAA-3′) of the mouse AR mRNA sequence (Bioneer, Daejeon, Korea). After 48 h, the transfected and control cells were treated with 25 μM acrolein (Sigma-Aldrich Co., St. Louis, MO) or 50 mU/ml glucose oxidase (GOX; Sigma-Aldrich), which generates ROS in the culture medium, for 5 h in the presence or absence of 50 μM ethyl 1-benzyl-3-hydroxy-2-(5*H*)-oxopyrrole-4-carboxylate (EBPC), a specific AR inhibitor (Tocris Cookson, Avonmouth, UK).

Determination of cell viability

To analyse cell viability using the trypan blue exclusion method, cells washed with ice-cold phosphate-buffered saline (PBS) were harvested and mixed with a 0.4% trypan blue solution. Viable cells in the cell suspension were counted with a hemacytometer and a lactate dehydrogenase (LDH) release assay was performed to determine cell viability. The amount of LDH released into the medium was determined by a CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, Madison, WI). The optical density of the coloured product was measured at 490 nm, as suggested by the manufacturer, using an Infinite 200 microplate reader (TECAN Austria GmbH, Grödig, Austria).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay

J774A.1 cells were seeded on coverslips in a six-well plate and incubated until 80% confluence was reached. The cells were then treated with the reagents described above. Cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS overnight at 4°C. The fixed cells were washed with 0.1% Tween-20 in PBS (PBS-T) and permeabilized for 90 min at 37°C in PBS containing 0.5% Triton X-100. Apoptotic cells were determined by a TUNEL assay using an *in situ* cell death detection kit (Roche Applied Science, Penzberg, Germany) for 90 min at 37°C. After staining, the cells were washed for 5 min in PBS-T three times and stained in a propidium iodide solution [10 mM Tris–HCl (pH 8.0), 1 mM NaCl, 0.1% NP-40, 0.7 μg/ml RNase A and 0.05 mg/ml propidium iodide] for 20 min at 37°C. After washing with PBS-T for 5 min, the cells were observed using confocal laser scanning microscopy.

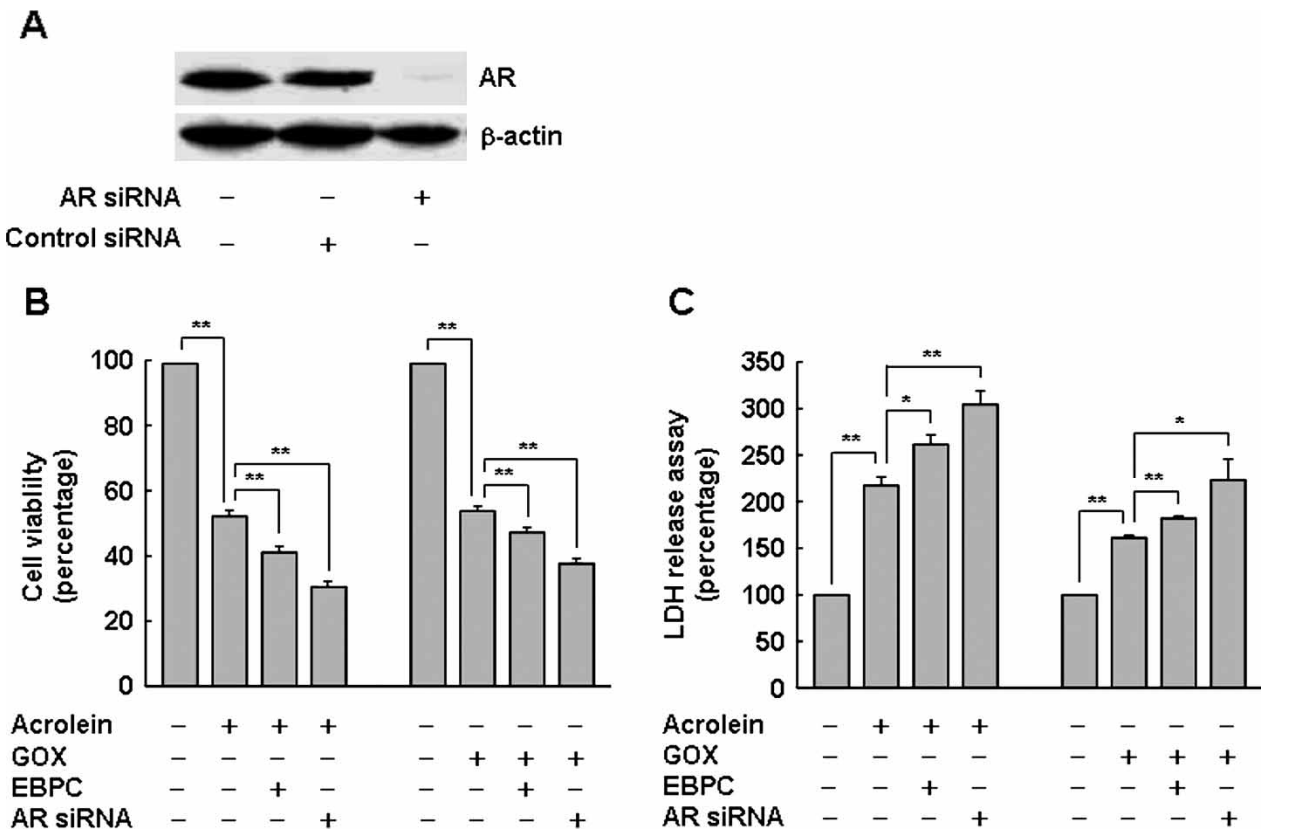

Figure 1. Effects of the down-regulation or inhibition of AR on acrolein- or GOX-induced cellular injury. (A) J774A.1 cells were transfected with 100 nM AR siRNA or scrambled control siRNA. After 48 h incubation, the cell lysates were immunoblotted with an anti-AR antibody or an anti-β-actin antibody. (B and C) J774A.1 cells transfected with an AR siRNA were treated with 25 μM acrolein or 50 mU/ml GOX for 5 h in the presence or absence of 50 μM EBPC, a specific inhibitor of AR. Cell viability was determined by trypan blue exclusion (B) or LDH release (C) assays. * $p<0.05$, ** $p<0.01$. Vertical columns represent the means ± SE ($n=4$).

TUNEL-positive cells were counted in at least four randomly-chosen fields.

Identification of apoptosis by fluorescence-activated cell sorting (FACS)

J774A.1 cells cultured in a 60-mm dish were treated with the indicated reagents, as described above. The cells were pelleted by centrifugation, washed with ice-cold PBS and fixed in 70% ethanol overnight. The cells were then washed with ice-cold PBS and stained in a propidium iodide solution. Following incubation in the dark for 30 min at room temperature, cell cycle profiles were determined using a FACS Calibur (Becton Dickinson Biosciences, San Jose, CA). At least 20 000 cells were analysed in each sample.

Measurement of ROS

J774A.1 cells treated with the indicated reagents were incubated with 10 μm dichlorofluorescin diacetate (DCFH-DA; Calbiochem, San Diego, CA) for 30 min, then harvested and washed twice with ice-cold PBS. The cells were immediately analysed for fluorescence intensity using a FACSCalibur (Becton Dickinson Biosciences). For analysis using confocal laser scanning microscopy, cells treated as described above were incubated with 10 μM DCFH-DA for 30 min and immediately observed using a confocal laser scanning microscope. ROS-generating cells were counted from at least four randomly-chosen fields.

Assessment of reactive aldehydes

Reactive aldehyde generation was assessed by measuring thiobarbituric acid reactive substances (TBARS), as described previously [23]. J774A.1 cells treated with the indicated reagents were washed with ice-cold PBS, collected and resuspended in 1 ml of thiobarbituric acid (0.4%) dissolved in 50 mM Tris buffer (pH 7.4) containing 180 mM KCl, 10 mM EDTA and 20 mM HCl. The samples were sonicated and heated at 95°C for 20 min. After the addition of 1 ml of butan-1-ol, the mixture was centrifuged at 2000 × g for 10 min at 4°C. The absorbance of the upper layer at 548 nm was measured using a spectrophotometer.

Preparation of cytosolic and mitochondrial cell extracts

J774A.1 cells seeded at a density of 5×10^5 cells in a 100-mm dish were treated with the reagents indicated above. Cells were washed twice in ice-cold PBS and resuspended in S-100 buffer [20 mm HEPES

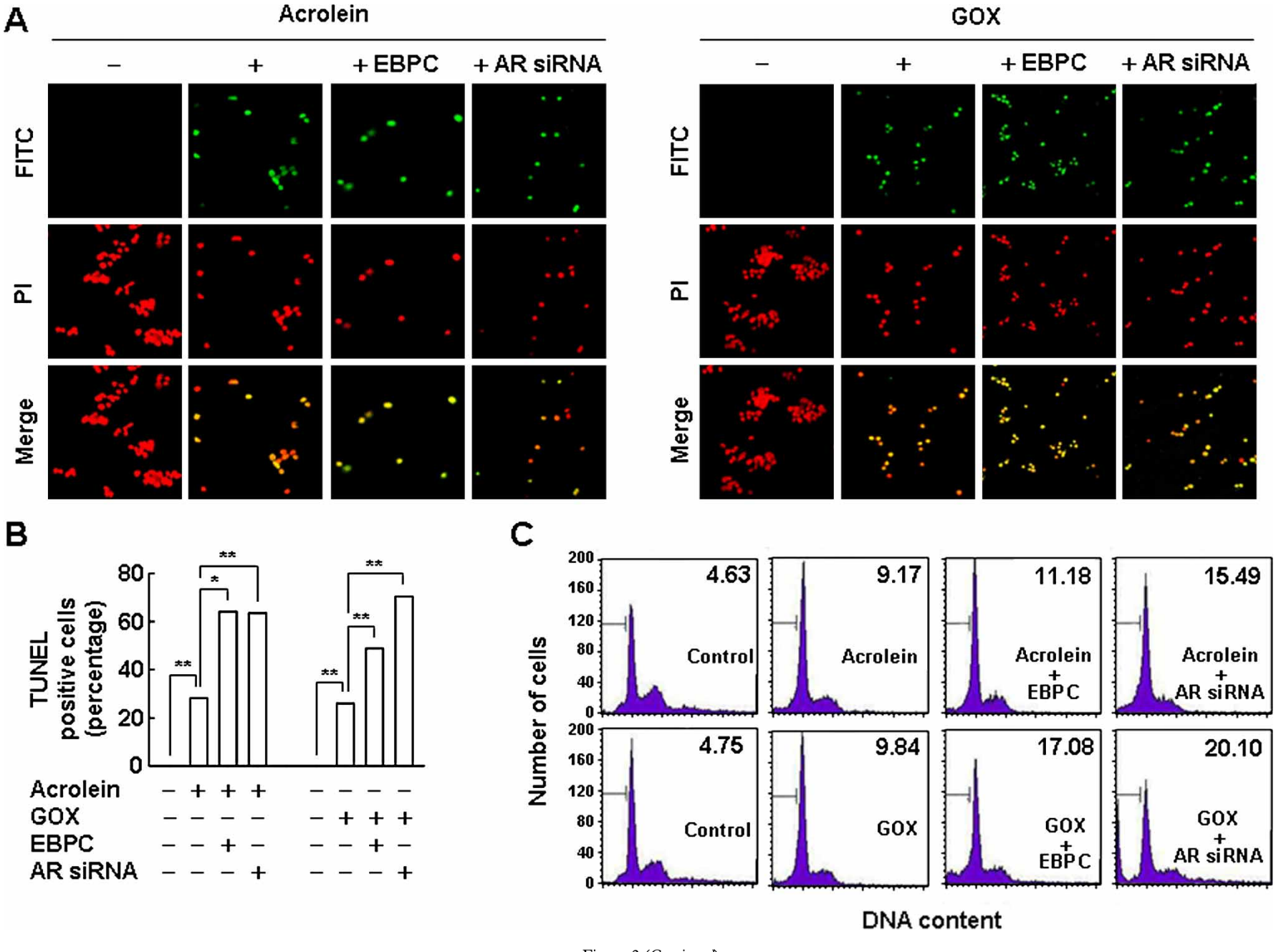

Figure 2 (*Continued*)

(pH 7.5), 10 mM KCl, 1.9 mM $MgCl_2$, 1 mM EGTA and 1 mM EDTA] containing a mixture of protease inhibitors. After incubation on ice for 20 min, the cells were homogenized with a glass dounce homogenizer. The homogenate was subjected to centrifugation at 1000 × g for 5 min to remove unbroken cells, nuclei and heavy membranes. The resulting supernatant was subjected to centrifugation again at 14 000 × g for 30 min to collect the mitochondria-rich and cytosolic fractions. The mitochondria-rich fraction was washed once with S-100 buffer, then was resuspended in lysis buffer [150 mM NaCl, 50 mM Tris-Cl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate and 1 mM EGTA] containing protease inhibitors. For isolation of total cell extracts, the cells were washed with ice-cold PBS and lysed in a PRO-PREP Protein Extraction Solution (iNtRON Biotechnology, Seoul, Korea) for 90 min at −20°C. The cell lysates were centrifuged at 10 000 × g for 20 min and the supernatants (total cell extracts) were collected.

Western blot analysis

A protein aliquot was subjected to SDS-polyacrylamide gel electrophoresis and transferred onto Hybond-P^+ polyvinylidene difluoride membranes (Amersham Biosciences). Membranes, blocked with 5% non-fat milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 for 2 h at room temperature, were reacted at 4°C overnight with the indicated primary antibodies in TBS containing 1% BSA and 0.05% Tween-20. The membranes were then incubated with peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (1:5000) for 1 h at room temperature. After washing in TBS containing 0.1% BSA and 0.1% Tween-20, the immunoreactive proteins were detected using WEST-ZOL Plus (iNtRON Biotechnology).

Statistical analysis

A Student's *t*-test was applied to compare the means. All data are expressed as means ± SE.

Results

Effects of AR down-regulation or inhibition on the viability of J774A.1 cells exposed to acrolein or GOX

To elucidate the role of AR in cellular damage following the exposure of cells to acrolein or GOX, the gene silencing of AR was carried out in J774A.1 macrophages. In contrast to the abundant expression of AR in control macrophages, AR levels were markedly reduced when cells were transfected with an siRNA against murine AR, but not when they were transfected with a control siRNA consisting of pooled non-specific sequences (Figure 1A). Trypan blue exclusion (Figure 1B) and LDH release (Figure 1C) assays revealed that siRNA-mediated AR down-regulation significantly enhanced the decline in the viability of J774A.1 cells exposed to acrolein or GOX. We performed the same experiments using EBPC, a specific inhibitor of AR, and observed similar results (Figure 1B and C). These data suggest that AR has a protective role in acrolein- or GOX-induced cellular injury.

Effects of AR down-regulation or inhibition in acrolein- or GOX-induced cell death

J774A.1 cells were next treated with acrolein or GOX and analysed by a TUNEL assay. The number of TUNEL-positive cells was increased following acrolein or GOX treatment (Figure 2A and B). This increase in apoptosis was further enhanced in the presence of EBPC or an siRNA against AR. These results suggest the anti-apoptotic role of AR in cells exposed to toxic substances. To further confirm our findings, a FACS assay was performed and revealed a marked increase in apoptotic cell death in cells exposed to acrolein or GOX (Figure 2C). The proportion of apoptotic cells was further amplified by the inhibition of enzyme activity or AR gene silencing. Collectively, these data suggest a protective role for AR in acrolein- or GOX-induced apoptosis.

Effects of AR down-regulation or inhibition on acrolein- or GOX-induced ROS generation

To investigate whether ROS are involved in acrolein- or GOX-induced apoptosis, we examined ROS levels in the presence or absence of EBPC or an AR siRNA. Acrolein and GOX elicited a significant increase in intracellular peroxide levels, which were further elevated in cells treated with an AR siRNA or EBPC (Figure 3). These results indicate that AR sequesters intracellular peroxides to protect against acrolein- or GOX-induced cell damages.

Effects of AR down-regulation or inhibition on the acrolein- or GOX-induced generation of thiobarbituric acid reactive substance (TBARS)

We next examined the generation of reactive aldehydes by measuring TBARS levels. J774A.1 cells

Figure 2. Effects of AR down-regulation or inhibition in acrolein- or GOX-induced cell death. (A) TUNEL staining of J774A.1 cells transfected with an AR siRNA and treated with acrolein or GOX for 5 h in the presence or absence of EBPC. Quantitative analyses for the TUNEL-positive cells were performed (B). Cells chosen from at least four randomly selected fields were scored for a TUNEL-positive reaction ($n = 100$ each). Values are the means ± SE from three separate determinations. * $p < 0.05$, ** $p < 0.01$. (C) FACS analysis of apoptosis. J774A.1 cells were treated with the indicated reagents and the population of sub-G_1 was detected with a FACS Calibur.

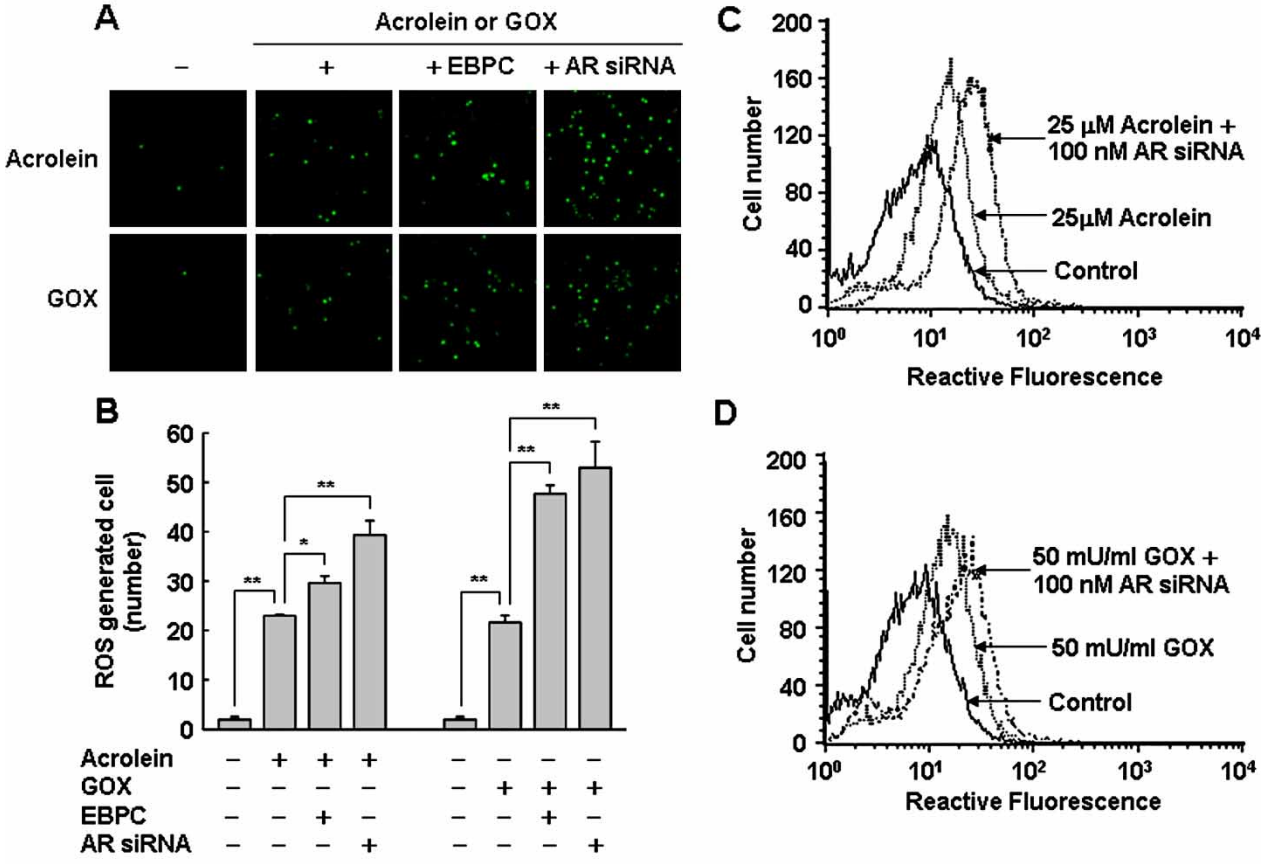

Figure 3. Effects of AR down-regulation or inhibition on acrolein- or GOX-induced ROS generation. J774A.1 cells transfected with an AR siRNA were treated with acrolein or GOX for 5 h in the presence or absence of EBPC. Cells were subsequently incubated with 10 μM DCFH-DA for 30 min at room temperature. ROS generation was visualized (A) and quantified (B) using fluorescence microscopy and was also characterized by a FACS Calibur (C and D). Values from at least four random fields are shown as the means ± SE. The results are representative of two to three independent experiments. * $p < 0.05$, ** $p < 0.01$.

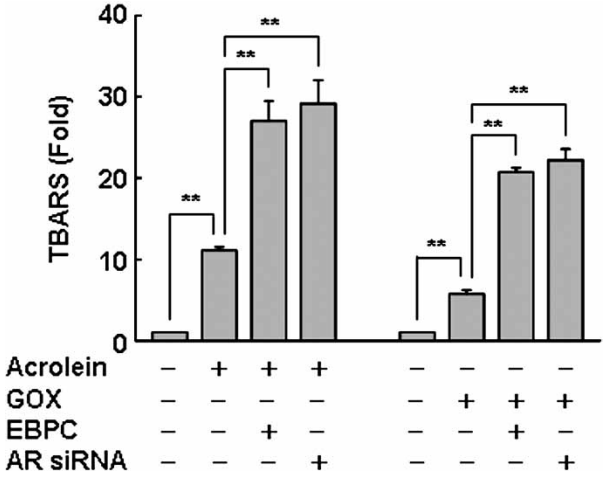

Figure 4. Effects of AR down-regulation or inhibition on acrolein- or GOX-induced reactive aldehyde generation. J774A.1 cells transfected with an AR siRNA were treated with acrolein or GOX for 5 h in the presence or absence of EBPC. Levels of reactive aldehydes were estimated by measuring TBARS levels. * $p < 0.05$, ** $p < 0.01$. Vertical columns represent the means ± SE ($n = 3$).

exposed to acrolein or GOX showed significantly increased TBARS levels (Figure 4), indicative of membrane lipid peroxidation [23]. TBARS levels were further augmented in cells treated with an AR siRNA or with EBPC, indicating that the generation of TBARS induced by acrolein or GOX is modulated by AR activity.

Effect of AR down-regulation or inhibition on the acrolein- or GOX-induced activation of apoptotic proteins

To further clarify the molecular mechanisms underlying the enhanced apoptotic cell death in AR siRNA- or EBPC-treated cells, changes in the levels of key apoptotic proteins were investigated in J774A.1 cells following their exposure to acrolein or GOX. Mitochondrial translocation of Bax was enhanced in cells treated with acrolein or GOX and was further augmented in the presence of EBPC or in cells transfected with an AR siRNA (Figure 5). In contrast, Bcl-2 levels in the mitochondrial fraction were attenuated in the presence of EBPC or in cells transfected with an AR siRNA. Cytochrome c release

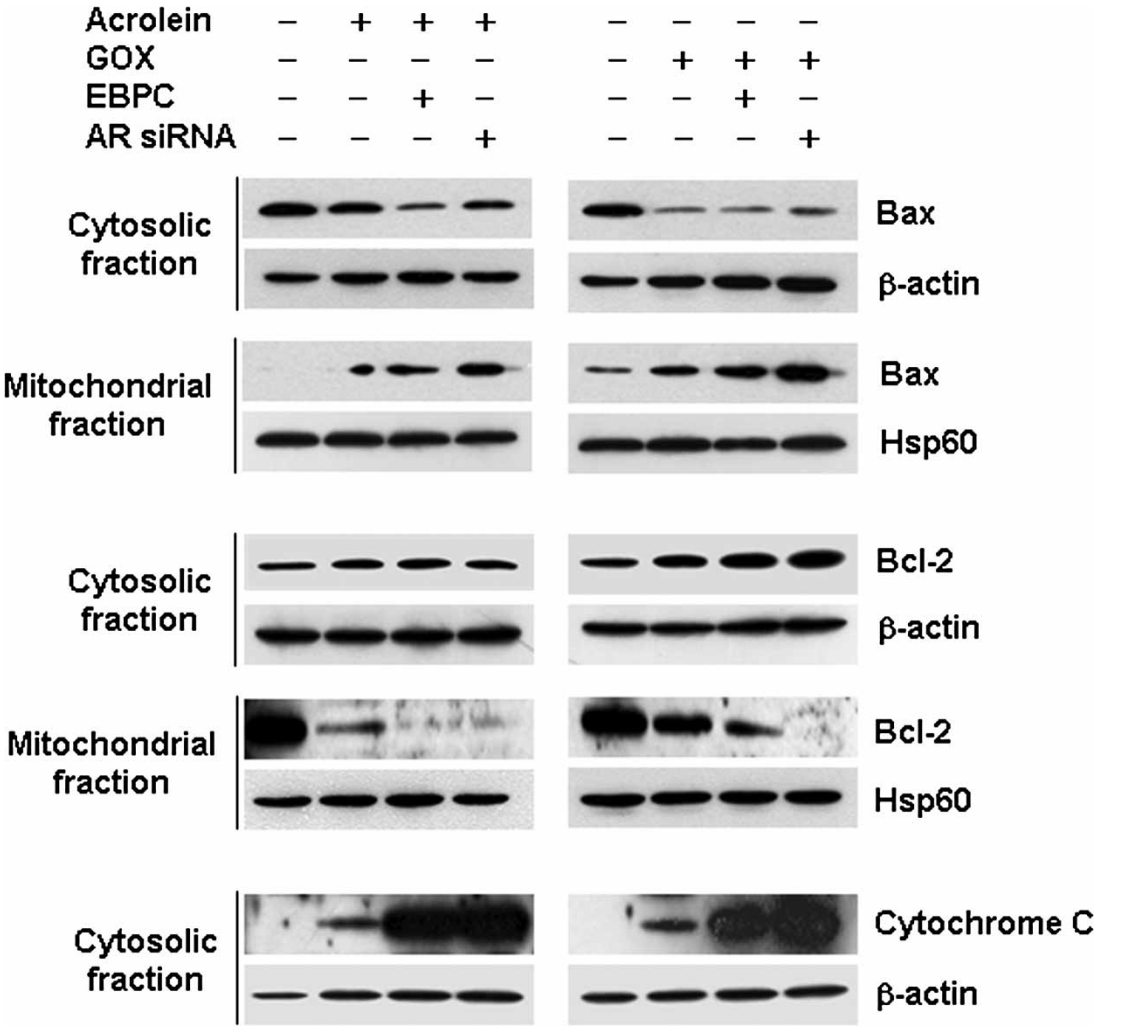

Figure 5. Effects of down-regulation or inhibition of AR on the acrolein- or GOX-induced activation of apoptotic proteins. J774A.1 cells transfected with an AR siRNA were treated with acrolein or GOX for 5 h in the presence or absence of EBPC. The levels of Bax, Bcl-2 and cytochrome c in the cytosolic and mitochondrial fractions were analysed by Western blot. Data are representative of two to three independent experiments.

into the cytosolic fraction was also detected in cells exposed to acrolein or GOX and increased amounts of cytochrome c were detected in the cytosolic fractions of EBPC- and AR siRNA-treated cells. These results suggest that AR plays an important role in resistance to acrolein- or GOX-induced cell death, through the regulation of key apoptotic proteins.

Discussion

In the present study, we demonstrated that the gene silencing of AR with an siRNA or the inhibition of AR activity rendered J774A.1 cells more susceptible to acrolein- or oxidative stress-induced cell death. AR down-regulation significantly accelerated the acrolein- or ROS-induced activation of apoptotic proteins, indicating a critical role for AR in cellular responses to oxidative stress-induced injury.

Down-regulation or inhibition of AR activity enhanced the susceptibility of macrophages to cellular injury. Since acrolein is a substrate of AR [18], AR may function by sequestering the reactive aldehydes generated during lipid peroxidation-mediated cell damage. In fact, the functional significance of AR against cytotoxic aldehydes and hydrogen peroxide has been documented in hepatoma cell lines [24], vascular smooth muscle cells [11,15,16,20] and vasculitis lesions [14]. Our present findings thus provide further insight into the primary role of AR in degrading the toxic molecules generated under oxidative stress.

Ablation of AR with an siRNA or inhibition of AR activity markedly accelerated the subsequent activation of apoptotic pathways. We observed the enhanced release of cytochrome c into the cytosolic fraction and a reduced level of Bcl-2 in the mitochondrial fraction of macrophages treated with an AR inhibitor or with an AR siRNA. Previous reports have demonstrated that ROS and acrolein elicit the

activation of the mitochondrial pathway [25,26]. This pathway involves changes in the mitochondrial membrane potential, translocation of Bcl-2 family proteins and cytochrome *c* release into the cytosol [27]. The involvement of oxidative stress in acrolein-induced toxicity has also been suggested in pneumocytes [28], epithelial cells [29] and in PC12 cells with significant cellular ROS accumulation [30]. Accordingly, reduced generation of ROS may block the subsequent activation of apoptotic pathways.

In contrast with our findings using J774A.1 cells, AR inhibition has been shown to prevent the RAW264.7 macrophage death caused by hydroxynonenal, an AR substrate derived from lipid peroxidation [31]. The reason for this discrepancy in the effect of AR inhibition is unclear. Our initial attempts to down-regulate AR levels in RAW264.7 cells were frustrated by the ineffectiveness of the transfected siRNA, due to the abundant expression of AR in this cell line. Still to be considered are the use of growth-arrested cells and the length of exposure to toxic aldehydes. This previous study maintained RAW264.7 cells in 0.1% FCS during an incubation with hydroxynonenal for 24 h; here, we maintained J774A.1 cells in 10% FCS during an incubation with acrolein for 5 h. The validity of these differences should be studied *in vivo* using animal models.

Activated macrophages elevate antioxidant levels to alleviate the damages evoked by intracellular oxidants [32,33]. Frequent exposure to toxic substances eventually induces self-defense mechanisms to attenuate cellular injury. It can therefore be assumed that activated macrophages express AR to protect themselves from the inwardly-directed damages induced by reactive molecules generated inside the cell. Collectively, our present findings shed light on the role of AR in cellular protection. These findings also lead to a better understanding of the self-defense mechanisms of macrophages, which are frequently exposed to reactive and toxic substances.

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***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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