Proteomic Analysis of Oxidative Stress-resistant Cells

A SPECIFIC ROLE FOR ALDOSE REDUCTASE OVEREXPRESSION IN CYTOPROTECTION*

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We are using a proteomic approach that combines two-dimensional electrophoresis and tandem mass spectrometry to detect and identify proteins that are differentially expressed in a cell line that is resistant to oxidative stress. The resistant cell line (OC14 cells) was developed previously through chronic exposure of a parent cell line (HA1 cells) to increasing hydrogen peroxide concentrations. Biochemical analyses of this system by other investigators have identified elevated content and activity of several classical antioxidant proteins that have established roles in oxidative stress resistance, but do not provide a complete explanation of this resistance. The proteomics studies described here have identified the enzyme aldose reductase (AR) as 4-fold more abundant in the resistant OC14 cells than in the HA1 controls. Based on this observation, the role of AR in the resistant phenotype was investigated by using a combination of AR induction with ethoxyquin and AR inhibition with Alrestatin to test the cytotoxicity of two oxidation-derived aldehydes: acrolein and glycolaldehyde. The results show that AR induction in HA1 cells provides protection against both acrolein- and glycolaldehyde-induced cytotoxicity. Furthermore, glutathione depletion sensitizes the cells to the acrolein-induced toxicity, but not the glycolaldehyde-induced toxicity, while AR inhibition sensitizes the cells to both acrolein- and glycolaldehyde-induced. These observations are consistent with a significant role for AR in the oxidative stress-resistant phenotype. These studies also illustrate the productive use of proteomic methods to investigate the molecular mechanisms of oxidative stress.

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Oxidative stress has been described as a component of numerous human diseases, including atherosclerosis, cancer, eye diseases such as age related macular degeneration, neurological diseases such as Alzheimer’s disease, and aging (1–5). Oxidative stress is characterized by the production of a diverse group of reactive species, known collectively as reactive oxygen species (ROS) and/or reactive nitrogen species (RNS), which can be generated by a number of pathways. For example, the premature release of partially reduced species from cytochrome oxidase is a significant source of ROS in mitochondria (6). Activated phagocytes also release significant amounts of ROS while attacking microorganisms via the release of superoxide (O₂⁻) by NADPH oxidase activity (7). Other biologically significant sources of ROS include ionizing radiation, xanthine oxidase activity, cytochrome p450 activity, and metal catalyzed reactions, to name a few (8–11). The RNS include nitric oxide, formed by nitric oxide synthase, nitrogen dioxide, formed by nitric oxide synthase and myeloperoxidase, and peroxynitrite, formed by the reaction of nitric oxide and superoxide (12, 13).

The production of ROS and RNS can, in turn, lead to the formation of a broad variety of other modified and oxidized cellular molecules. These damaged cellular components can have inherent toxicity and/or may degrade to form additional levels of toxic products. Superoxide, for example, forms hydrogen peroxide that can subsequently be used by peroxides to form hypohalous acids such as HOCI and HOBr or be reduced by Fe²⁺ or Cu⁺ to form a hydroxyl radical (OH*) (14, 15). Lipid peroxidation and amino acid oxidation can result in the production of a variety of toxic aldehydes. 4-hydroxy-2-nonenal (4HNE) and acrolein are examples of α,β-unsaturated aldehydes, a highly reactive class of aldehydes that are formed as a result of the degradation of peroxidized lipids (16, 17), whereas acrolein, 2-hydroxy-propanal, and glycolaldehyde are examples of aldehydes derived from amino acid oxidation (18).

The damage to biological system that the products of ROS and RNS create is based on their ability to covalently react with and modify other cellular molecules to alter biological function, including protein, lipid, and DNA modifications. Be-
cause of the potential harm of these species, a number of cellular defenses are in place to minimize the extent of either the production or damage. Well-described antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase are able to metabolize superoxide, hydrogen peroxide, or fatty acid hydroperoxides, respectively, in order to minimize the effects of their production. Other enzymes such as glutathione S-transferase may also be considered a part of the antioxidant protection system because of their ability to metabolize other products of ROS and RNS production (i.e. α,β-unsaturated aldehydes) (19).

One might infer, based on this diverse set of products and pathways, that an extensive system of protective pathways is needed to protect cells and tissues from oxidative stress. Therefore, in order to further expand our understanding of cellular defenses against oxidative stress, we have initiated a proteomic evaluation of a mammalian cell line that was developed as a model of oxidative stress resistance (20). Constant exposure of a Chinese hamster fibroblast cell line (HA1) to progressive concentrations of hydrogen peroxide yielded a strain (designated OC14) with a stable and permanent oxidative stress-resistant phenotype. Changes that have been characterized to date include a 20-fold increased catalase activity compared with the parent cell line HA1 (20) that is accompanied by elevated steady-state catalase mRNA and protein levels (21), elevated glutathione peroxidase and Cu/Zn-superoxide dismutase (SOD) activities (22), and elevated glutathione levels (23). It is interesting to note that these OC14 cells, which have been produced by hydrogen peroxide treatment, are also resistant to 95% oxygen, a number of aldehydes, nitric oxide, heat, and cisplatin (22–27).

We are now using proteomic methods to further the characterization of this oxidative stress-resistance phenotype. The experiments are using two-dimensional (2D) gel electrophoresis to separate the total cellular proteins for the OC14 cells relative to the HA1 cells. This proteomic survey is systematically identifying and characterizing the proteins that are differentially expressed in this system. The long-term goal of these experiments is to describe new mechanisms of oxidative stress resistance that can be used to manipulate the damaging effects of ROS and RNS in human disease. In this report, we focus on aldose reductase (AR), an enzyme that is part of the polyol pathway but is also capable of detoxifying aldehydes by reduction to the corresponding alcohol. This protein was found in our proteomic experiment to be overexpressed in the OC14 cells. Our results show that inducing a comparable overexpression in the HA1 cells provides a significant protection against both acrolein and glycolaldehyde toxicity that can be reversed by AR inhibition. These results provide a rational explanation for the known resistance of the OC14 cells to aldehyde-induced cytotoxicity. Furthermore, these results underscore the ability of 2D electrophoresis-based proteomic experiments to reveal significant, phenotype-related differences in cellular systems as leads for new studies.

**EXPERIMENTAL PROCEDURES**

**Materials**—The 2D electrophoresis materials, including 11-cm pI 5–8 immobilized pH gradient (IPG) gels, the detergent-compatible protein assay kit, and amphotoles were purchased from Bio-Rad Corporation (Hercules, CA). The Coomassie stain, GelCode Blue, was purchased from Pierce Chemical Co. (Rockford, IL). The CellTiter96 cell viability assay kit and sequencing-grade, modified trypsin was purchased from Promega (Madison, WI). The Alrestatin was purchased from Tocris Corporation (Ellisville, MO). The reagents used in the reverse transcriptase (RT)-PCR experiments were purchased from Tel-Test (TriZOL; Friendswood, TX) and Applied Biosystems (Taqman reagents; Foster City, CA). The primers were ordered from Integrated DNA Technologies (Coralville, IA). All other reagents were purchased from Sigma Chemical Corporation (St. Louis, MO). The capillary columns used in the liquid chromatography (LC) mass spectrometry (MS) analyses were slurry-packed in our laboratory with Phenomenx Jupiter C18 material (Torrance, CA) packed in a 50-μm i.d. PicoFrit electrospray tip from New Objective (Woburn, MA).

**Cell Culture, Drug Treatment, and Toxicity Assay**—Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/liter glucose, glutamine, penicillin-streptomycin, and 10% fetal calf serum (FCS) at 37 °C in 5% CO2. For the AR induction, the cells were treated overnight with 50 μM ethoxyquin in DMEM with 10% FCS. The induced cells were harvested with trypsin, replated in 24-well plates at a density of 35,000 cells per well, and allowed to attach and grow over a second night prior to the toxicity testing. Uninduced controls were handled in parallel without the ethoxyquin induction. For the AR inhibition, cells were plated in 24-well plates at a density of 35,000 cells per well, allowed to attach and grow overnight, and pretreated with 1 mM Alrestatin in DMEM with 5% FCS for 2 h prior to aldehyde treatment. For the glutathione depletion, cells were plated in 24-well plates at a density of 35,000 cells per well, allowed to attach, and treated overnight with 1 mM buthionine sulfoximine (BSO) in DMEM with 10% FCS.

For the toxicity testing, cells plated and prepared as described above were treated on the following day with assay medium containing the desired concentration of the respective aldehydes for 2 h. In the AR inhibition and glutathione depletion experiments, the Alrestatin and/or BSO were maintained during the aldehyde treatments. After the treatment, the media was replaced with DMEM and 10% FCS and cells are incubated overnight. Cell viability was assayed by replacing the media with the chromogenic reagent CellTiter96 and reading the absorbance at 490 nm after 45 min at 37 °C. All values were corrected for a no-cell blank and normalized to controls treated with the Alrestatin and BSO, where appropriate, but not treated with aldehyde.

**2D Electrophoresis and Protein Identification (28)**—Cells monolayers were harvested with trypsin, washed two times with phosphate-buffered saline containing a protease inhibitor mixture (Complete mini protease inhibitor tablets; Roche, Indianapolis, IN), and lysed in 25 mM Tris, pH 7.5, 2.5 mM MgCl2, 0.5% SDS, heated to boiling for 5 min. Lysates were cooled and treated with 50 μg/ml DNase I, 50 μg/ml RNase A for 15 min. Protein concentration in the homogenate was determined with a SDS-compatible assay (Bio-Rad DC protein assay) prior to precipitation with acetone (80% w/v). The protein precipitates were resuspended in a solubilization buffer containing 7 M urea, 2 M thiourea, 1% dithiothreitol, 1% 3-[G-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid, 1% amphotoles (BioLyte 3/10), and 1% Triton X-100. The 11-cm IPG strips (pI 5–8) were rehydrated in 180-μl aliquots containing 750 μg of protein. The isoelectric focusing program accumulated 60 kVh overnight. Focused strips were then equilibrated, with reduction and alkylation, in a buffer containing 6 M urea, 2% SDS, 20% glycerol, and either 100 mM dithiothreitol (for the reduction) or 100 mM iodoacetamide (for the alkylation). The equilibrated strip was immediately subjected to SDS-PAGE for the second
dimension in a 12.5% gel (Criterion gel). The gels were fixed with 50% ethanol, 10% acetic acid, rinsed with water 3 times, and stained overnight using a colloidal Coomassie blue stain (GelCode Blue). Stained gels were washed with water and recorded by scanning.

For the protein identification, selected gel bands were excised, dehydrated with acetonitrile, rehydrated with 50 mM ammonium bicarbonate, and dehydrated again in acetonitrile. After drying in a SpeedVac, the trypsin (20 μg/ml in 50 mM ammonium bicarbonate) was incorporated into the bands by reswelling on ice for 10 min. The excess trypsin solution was removed and 5 μl of 50 mM ammonium bicarbonate added to submerge the band and maintain hydration. After an overnight incubation at 37°C, the tryptic peptides that were formed were extracted from the band with 2–30 μl aliquots of 50% acetonitrile/10% formic acid. The extract was concentrated in a SpeedVac to 0.5 μl and 20 μl with 1% acetic acid added to give a final volume of 25 μl.

The digests were analyzed by capillary column LC tandem MS using a 50-μM i.d. × 6-cm-long Phenomenex Jupiter C18 capillary column eluted with a linear gradient of acetonitrile in 50 mM acetic acid (2–70% acetonitrile in 45 min) at a flow rate of 200 nl/min. The MS system was a ThermoFinnigan LCQ-deca ion trap mass spectrometer (San Jose, CA). The instrument was operated in the data-dependent mode in which one mass spectrum and three collision-induced dissociation (CID) spectra were acquired per cycle as described (28). The LC-tandem MS data were analyzed using the program TurboSequest to search all the CID spectra in each dataset versus the National Center for Biotechnology Information (NCBI) non-redundant database. All database matches were verified by manual inspection of the matching spectra to verify proper interpretation of the CID spectra.

Preparation of RNA and Quantitative RT-PCR—RNAzol was used to prepare RNA. Cells were rinsed with phosphate-buffered saline, lysed on the plate with RNAzol reagent, and prepared as directed. RNA was quantitated spectrophotometrically by absorption at 260 nm, and Taqman reverse transcription reagents were used to generate PCR template. The quantitative PCR experiments were conducted using and Applied Biosystems Prizm 9600 in reactions containing 0.5 μM each primer, using SYBR Green PCR core reagents as recommended by the manufacturer. Hamster AR forward primer nt696–716 and reverse and complement primer nt845–821 of NCBI accession no. U81045 were used for AR message quantitation. Hamster glyceraldehyde-3-phosphate dehydrogenase primers were designed for mRNA control using an nt685–107 forward primer and an nt685–608 reverse and complement primer based on NCBI accession no. X52123. For quantitation of AR mRNA levels, cycle numbers were normalized using the glyceraldehyde-3-phosphate dehydrogenase amplification curves.

RESULTS

AR Identification—Fig. 1 shows representative gels from a series of 2D gel electrophoresis experiments analyzing total cell lysates from HA1 (A) and OC14 (B) cells. The isoelectric focusing step used 750 μg of protein isolated from actively growing adherent cultures and incorporated in the IPG strip by active rehydration overnight at 50 V. The isoelectric focusing step accumulated ~60,000 Vh. The strips were reduced and alkylated, and molecular mass separation was carried out in a 12.5% SDS-PAGE gel for ~1 h at 200 V. The gels were fixed and stained with a colloidal Coomassie blue. The boxed regions shown in B are enlarged in Figs. 2 and 3.

FIG. 1. 2D gel electrophoresis of total cell lysates from HA1 (A) and OC14 (B) cells. The isoelectric focusing step used 750 μg of protein isolated from actively growing adherent cultures and incorporated in the IPG strip by active rehydration overnight at 50 V. The isoelectric focusing step accumulated ~60,000 Vh. The strips were reduced and alkylated, and molecular mass separation was carried out in a 12.5% SDS-PAGE gel for ~1 h at 200 V. The gels were fixed and stained with a colloidal Coomassie blue. The boxed regions shown in B are enlarged in Figs. 2 and 3.

FIG. 2. Regions of the HA1 (A) and OC14 (B) gels containing Cu/Zn SOD. The protein bands are indicated by the arrows in each region. These regions correspond to boxed region 1 in Fig. 1. The distinctly elevated Cu/Zn SOD seen in these gels for the OC14 is consistent with previous Western blot analysis and activity assays.

FIG. 3. Regions of the HA1 (A) and OC14 (B) gels containing AR. The protein bands are indicated by the arrows in each region. These regions corresponds to boxed region 2 in Fig. 1. The increased level of AR protein in the OC14 cells is established in this comparison.
protein homogenates from lysates of the HA1 and OC14 cells. These gels were loaded with 750 μg of total protein and ~1000 protein bands were detected with abundances ranging over nearly two orders of magnitude. Repetitive analyses of these homogenates gave similar and reproducible patterns of protein expression for each cell line, facilitating the visual identification of a number of differentially expressed proteins. Subsequent capillary column LC-tandem MS analyses permitted unambiguous identification of those proteins.

Two areas of the gels boxed in Fig. 1 are shown in more detail in Figs. 2 and 3. Fig. 2 shows a low molecular mass region of the gel extending from approximately pI 6.5 to 7.0. The band in Fig. 2 that is indicated by the arrows is more abundant in the OC14 cells than in the HA1 controls and was identified by MS as Cu/Zn SOD by matching to NCBI accession no. 20896095 (calculated molecular mass 15.9 kDa, calculated pI 6.0). Elevated expression of Cu/Zn SOD is consistent with previous Western analysis and activity assays (22). This result is presented as a general validation of the 2D methods, and the ability of these experiments to detect proteins known from other studies to be overexpressed as more abundant protein bands in these gels.

The gel detail shown in Fig. 3 contains a second region of the OC14 gel between pI 6.5 and 7.0, covering proteins with molecular masses between 45 and 60 kDa. The protein band indicated with the arrows is also significantly more abundant in the OC14 gel than in the HA1 control gel. Digestion and analysis of this band by LC-tandem mass spectrometry identified the protein as AR, corresponding to NCBI accession no. 2114406 (calculated molecular mass = 36.3 kDa, calculated pI = 6.2). As shown in Fig. 4, the LC-tandem MS analyses detected and sequenced peptides containing 172 amino acids covering 54% of the database sequence (29).

**Aldose Reductase in an Oxidative Stress-resistant Proteome**

| Amino Acid Sequence of AR | Amino Acid Sequence of AR |
|---------------------------|---------------------------|
| MSTFVELSTK               | GSNRNWRACL               |
| AKMPIVGLGT               | LFRTPVYVQIAL            |
| WQPPPGQVKR               | QPPQPLQPQK              |
| AKVKAIDAGY               | ELFPKDDQGN              |
| RHIDCAYAYY               | VLTSKITYLD             |
| NEHEVGEATQ               | LDVYLLK                |
| EKIKEKAVRR               | VCQKTPAR               |
| 71                        | 208.96095               |
| 81                        | 231.00184               |
| 81                        | 241.00171               |
| 101                      | 251.00181               |
| 111                      | 261.00191               |
| 121                      | 271.00201               |
| 131                      |                          |
| EDLPFVSKLM               | SLGSPNPWQ              |
| PTCFERKLLK               | APKEDPSLLE             |
| EAFQKTLTDL               | DPKIKEIAAK             |
| KLDYDLYLI                | HKKTSATQVLI            |
| HWPQLQPGK                | RHFIQRNVVY            |
| ELFPKDDQGN               | IPKVSTPAR              |
| VTQVECHPY                | HENFQVPDPQ             |
| LTQEKXIEYC               |                          |
| HSKGIVTAY                |                          |
| 141                      | 281.00191               |
| 151                      | 291.00191               |
| 161                      | 301.00191               |
| 171                      | 311.00191               |

**Fig. 4. Amino acid sequence of AR.** The underlined portions of the sequence represent peptides that were detected and sequenced in the mass spectrometric analyses.

**Fig. 5. Induction of AR by ethoxyquin treatment.** For the ethoxyquin induction, cells were plated and treated with 50 μM ethoxyquin. All cells were harvested simultaneously at the end of the treatment, with ethoxyquin treatment started at the indicated amount of time prior to harvest to ensure similar outgrowth from plating time. Cells for the t = 0 time point were not treated with ethoxyquin. RNA and protein were prepared from each of these samples and analyzed by quantitative RT-PCR and 2D electrophoresis. A shows the induction of AR at the protein level. B shows the accumulation of AR message. Fold induction of AR message is estimated from the number of cycles necessary for RT-PCR products to reach a threshold level, using GAPDH message as an internal control for normalization.

was used in our experiments to induce AR in the HA1 cells. Fig. 5A shows the time course induction of AR protein in the HA1 cells, determined by 2D electrophoresis. Quantitative RT-PCR, shown in Fig. 5B, confirms that increased mRNA levels precede AR induction. AR protein continues to accumulate and persists at 24 h, while the message level diminishes during the same period of time.

We assessed the functional effect of AR induction in these cells by measuring the cytotoxicity of acrolein and glycoalde...
controls (HA1). At acrolein concentrations greater than 10 μM, the toxicity of this class of aldehyde (33). Other biologically relevant compounds also represent different classes of aldehydes that have also been described as an advanced glycation end-product (34). The structure of glycoaldehyde is distinct from acrolein, being an α-hydroxyaldehyde rather than an α,β-unsaturated aldehyde. As a result, glycoaldehyde is an example of an aldehyde that does not readily react with sulfhydryls such as GSH. Detoxification of this class of aldehydes by AR cannot benefit from either the GSH conjugation or the Glutathione-depleting reagent BSO). However, ethoxyquin-induced AR did provide protection from acrolein toxicity at the 5-μM treatment level in this experiment. Subsequent focus on the sub-10-μM treatment range (Fig. 7) shows that AR induction protects the GSH-depleted HA1 cells compared with the GSH-depleted controls without AR induction. Furthermore, AR induction in the GSH-depleted HA1 cells gives a resistance to acrolein toxicity that is similar to the resistance seen in the GSH-depleted OC14 cells. These results suggest a significant role for AR in the detoxification of α,β-unsaturated aldehydes like acrolein and 4HNE, and that the availability of glutathione improves the protective effect of AR activity for this class of aldehyde.

**Glycoaldehyde Toxicity**—The second aldehyde tested was glycoaldehyde, which is generated through the oxidation of serine under conditions associated with oxidative stress (18). In addition, immunochemical data suggest that glycoaldehyde-modified proteins are present in the serum of diabetic patients (34). The structure of glycoaldehyde is distinct from acrolein, being an α-hydroxyaldehyde rather than an α,β-unsaturated aldehyde. As a result, glycoaldehyde is an example of an aldehyde that does not readily react with sulfhydryls such as GSH. Detoxification of this class of aldehydes by AR cannot benefit from either the GSH conjugation or the enhanced AR activity attained with glutathionylation.

The cytotoxicity of glycoaldehyde is shown in Fig. 8. It is significant to note that glycoaldehyde is considerably less toxic than acrolein, requiring an ~20-fold higher concentra-

![Acrolein Toxicity](image)

**Fig. 6.** Acrolein-induced cytotoxicity in HA1 and ethoxyquin-treated HA1 cells. For the survival assay, the cells were distributed in 24-well plates (30,000/well) and allowed to attach (at least 2 h). The complete media (DMEM with 10% FCS) was exchanged, either with or without the glutathione-depleting reagent BSO, and cells were grown overnight. Cells were then exposed to acrolein for 2 h by exchanging the media with DMEM (no serum) containing increasing concentrations of acrolein. Treatment was stopped by exchanging the treatment media with complete media for overnight outgrowth. Cell viability was then assayed by monitoring cellular respiration using the reagent Promega Celltiter96, diluted in DMEM. Normalized survival is reported as a percentage relative to the 0 μM-treated control in the same experiment. All concentrations were assay in triplicate, and the data are plotted as the mean ± S.D. Statistically significant differences were determined by analysis of variance using a Tukey-Kramer multiple comparison test and are indicated by * (HA1+E versus HA1) or # (HA1+BSO+E versus HA1+BSO).

![Acrolein Toxicity](image)

**Fig. 7.** Acrolein-induced cytotoxicity in HA1 and ethoxyquin-treated HA1 cells, focusing on the sub-10-μM treatment range. Toxicity assay was performed as described for Fig. 6, but with 0-, 2-, 4-, and 6-μM acrolein treatments. Statistically significant differences determined by analysis of variance using a Tukey-Kramer multiple comparison test are indicated by * (HA1+E+BSO versus HA1+BSO).
tion to produce 50% survival. Ethoxyquin pretreatment does provide a detectable protection, although the extent of protection is quantitatively smaller than that seen for acrolein-induced cytotoxicity. Not surprisingly, GSH depletion does not sensitize either the HA1 or OC14 cells to glycoaldehyde toxicity (data not shown). As with the acrolein treatment described above, the pattern of toxicity in the HA1/E cells was identical to the oxidative stress-resistant OC14 cells (data not shown).

**AR Inhibition**—To further clarify the importance of AR participation in aldehyde detoxification, we utilized one of the many AR inhibitors designed for therapeutic intervention in diabetes patients. Alrestatin binds preferentially to the enzyme/NADP+ complex, functioning as a noncompetitive inhibitor for aldehydic substrates that interferes with NADP+/NADPH exchange (35). Alrestatin is not one of the most potent AR inhibitors, but its inhibitor binding specificity is well characterized (36).

As shown in Fig. 9, Alrestatin pretreatment sensitizes HA1/E cells to acrolein toxicity. This reversal of the protective effect of the ethoxyquin treatment clearly shows that AR induction, as opposed to some nonspecific effect, is responsible for the protection. A similar sensitization is seen in Fig. 10 for glycoaldehyde toxicity with the Alrestatin pretreatment sensitizing the HA1 and HA1/E cells to the glycoaldehyde, directly supporting the role for AR in the ethoxyquin-induced protection.

**DISCUSSION**

The model system used in these experiments contains a demonstrable oxidative stress-resistant phenotype. The OC14 cell line, developed by chronic treatment of the HA1 cells with increasing concentrations of hydrogen peroxide, are resistant to several oxidative stresses including hydrogen peroxide, 95% oxygen, nitric oxide, and aldehydes (22–26). Proteomic analysis of OC14 cells, shown in Figs. 1–3,
identified a previously undescribed up-regulated protein, AR, in the oxidative stress-resistant OC14 cells.

AR [EC 1.1.1.21] has been found to have a broad substrate specificity, including the capability of converting numerous aldehydes to the corresponding alcohols. By comparing a variety of substrates, Vander Jagt et al. (37) showed that aldehydes containing an aromatic ring are excellent substrates, whereas other common small aldehydes, like formaldehyde and acetaldehyde, are poor substrates. Hydrophilic aldehydes are good substrates if the α carbon is oxidized, and glycolaldehyde is an example of this type of aldehyde. Important α,β-unsaturated aldehydes, like acrolein and 4HNE, were also shown to be good substrates for AR. AR is transcriptionally induced in response to 4HNE (38), and AR has been shown to catalyze the reduction of 4HNE in perfused rat aorta (39). In those experiments, ~20% of the perfused HNE was detoxified through conjugation to glutathione, with half of the GS-HNE subsequently reduced by AR to form GS-1,4-dihydropyridine-2-one. The improved AR activity toward GSH-conjugated 4HNE is due, at least in part, to a GSH binding site present on AR (40).

Our results show that AR induction by ethoxyquin provides significant protection of the HA1 cells to both acrolein- and glycolaldehyde-induced cytotoxicity. Quantitatively, the protective effect is greater for acrolein toxicity, increasing the 50% isosurvival dose by 75%, than for glycolaldehyde toxicity, where the 50% isosurvival dose is increased by 10% (Fig. 6 versus Fig. 8). This difference may simply reflect the high concentration of glycolaldehyde needed to produce toxicity and the inability of AR-catalyzed metabolism to significantly diminish this amount of glycolaldehyde in the time course of these experiments. However, the relative catalytic efficiency (kcat/Km) of the AR reaction with acrolein as substrate is better than the efficiency with glycolaldehyde as substrate (1.1 x 10^6 M^-1 min^-1 versus 9.5 x 10^6 M^-1 min^-1 (37), respectively), consistent with this relative protection. The protective effect for acrolein may also be enhanced by the enhanced catalytic efficiency for glutathione-conjugated aldehydes (40).

AR is implicated in the etiology of long-term diabetic complications. Briefly, AR catalyzes the reduction of glucose to sorbitol, representing the committed step into the polyol pathway. Sorbitol is then converted to fructose by sorbitol dehydrogenase. Under conditions of hyperglycemia, the second step in this pathway produces an excess of NADH, resulting in a chronically elevated cytosolic NADH/NAD+ ratio. Mitochondrial respiration is eventually unable to correct this redox imbalance, and the resulting condition has physiological consequences similar to hypoxia (ischemia, for instance). This sustained pseudohypoxia, and the associated consequences, has been proposed to have a pivotal role in the onset of long-term diabetic complications (41). Moreover, there is now evidence that overexpression of AR associated with specific genetic polymorphisms is also linked to elevated risk for diabetes complications (42, 43). The role for AR in diabetic complications is the rationale for the development of aldose reductase inhibitors (ARIs) that might prevent or delay these complications (44, 45).

Our data, however, shows that AR inhibition suppresses the protective effect of AR induction by ethoxyquin treatment for both acrolein and glycolaldehyde toxicity (Figs. 9 and 10). The protective effect of induced AR is consistent with the well-established enzymatic activity of the enzyme. The suppression of this protective effect by the AR inhibitor Alrestatin provides direct evidence that the protection seen with ethoxyquin treatment is due to increased AR activity. The ability of Alrestatin to sensitize cells to these aldehydes is also noteworthy considering the intended therapeutic use of AR inhibitors. Although AR activity may have a role in the pathology of diabetes, our experiments show that it provides an important detoxifying activity as well. Therefore, one might speculate based on these data that the use of ARIs in the treatment of diabetes can have the unintended consequence of increasing the availability of toxic aldehydes known to be produced in vivo. The role of AR in the detoxification of aldehydes might become more important as the therapeutic actions of new, more potent ARIs are tested, perhaps requiring the design of inhibitors that more specifically target the glucose-reducing activity while retaining the ability to reduce other aldehydes.

In summary, the results of these experiments establish a functional rationale for the presence of elevated AR in the oxidative stress-resistant OC14 cells and clarifies the potential role of AR as a detoxifying enzyme under conditions of oxidative stress. In adapting to growth with constant oxidative stress, OC14 cells were derived through permanent genomic changes in parental HA1 cells that supported an increase in the expression levels of a variety of well-characterized antioxidant enzymes in order to survive (catalase, Se-dependent glutathione peroxidase, Cu/Zn-SOD, etc.). Results presented here demonstrate that OC14 cells rely on AR to detoxify aldehydic toxins that can become significant under conditions of oxidative stress, with glutathione providing critical assistance in the AR-mediated detoxification of the more reactive, α,β-unsaturated aldehydes. These results also demonstrate the successful utilization of 2D gels to visualize differential expression patterns in this system. Additional changes in protein expression in the oxidative stress-resistant proteome of OC14 cells are currently under investigation.

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