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Human aldo–keto reductase AKR7A2 protects against the cytotoxicity and mutagenicity of reactive aldehydes and lowers intracellular reactive oxygen species in hamster V79-4 cells

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ABSTRACT

Aldo–keto reductase (AKR) enzymes are critical for the detoxication of endogenous and exogenous aldehydes. Previous studies have shown that the AKR7A2 enzyme is catalytically active toward aldehydes arising from lipid peroxidation, suggesting a potential role against the consequences of oxidative stress, and representing an important detoxication route in mammalian cells. The aim of this study was to determine the ability of AKR7A2 to protect cells against aldehyde cytotoxicity and genotoxicity and elucidate its potential role in providing resistance to oxidative stress. A transgenic mammalian cell model was developed in which AKR7A2 was overexpressed in V79-4 cells and used to evaluate the ability of AKR7A2 to provide resistance against toxic aldehydes. Results show that AKR7A2 provides increased resistance to the cytotoxicity of 4-hydroxynonenal (HNE) and modest resistance to the cytotoxicity of *trans*, *trans*-muconaldehyde (MUC) and methyglyoxal, but provided no protection against crotonaldehyde and acrolein. Cells expressing AKR7A2 were also found to be less susceptible to DNA damage, showing a decrease in mutation rate cause by 4-HNE compared to control cells. Furthermore, the role of the AKR7A2 enzyme on the cellular capability to cope with oxidative stress was assessed. V79 cells expressing AKR7A2 were more resistant to the redox-cycler menadione and were able to lower menadione-induced ROS levels in both a time and dose dependent manner. In addition, AKR7A2 was able to maintain intracellular GSH levels in the presence of menadione. Together these findings indicate that AKR7A2 is involved in cellular detoxication pathways and may play a defensive role against oxidative stress *in vivo*.

1. Introduction

Aldehydes and ketones are found in a range of compounds including drugs, food and environmental pollutants, and are also produced endogenously. Some aldehyde and ketones such as acrolein and 4-hydroxy *trans* 2-nonenal (4-HNE) are extremely reactive, and can cause damages to protein, DNA and lipids, leading to pathophysiological consequences [1]. For example, 4-HNE has been reported to cause cytotoxicity in several cell lines, and is the most toxic aldehyde produced during the peroxidation of ω-6-polyunsaturated fatty acids [2]. In addition, several reactive aldehydes have been confirmed as mutagenic through their interactions with DNA [3], and have been reported to induce mutations in mammalian cell lines in a dose-dependent manner [4]. These damaging events may play a significant role in the progression of diseases where elevation of oxidants is known to be high [5].

Other reactive aldehydes such as the benzene metabolite *trans*, *trans*-muconaldehyde (MUC) can cause DNA strand breaks leading to induce bone marrow depression and inhibition of erythropoiesis in mice [6]. Another reactive aldehyde, methyl glyoxal, is an active intermediate in the Maillard reaction, cross-linking with proteins. It is elevated in uncontrolled diabetes, and is associated with diabetic complications [7].

In order to counter the potentially lethal effects of toxic aldehydes and ketones, several metabolizing enzyme systems exist that can render aldehydes either more readily excreted or less biologically active. Aldo–keto reductases (AKR) are a superfamily of NADP(H)-dependent enzymes that reduce aldehydes and ketones to alcohols [8]. The AKR superfamily includes enzymes from bacteria, yeast, plants and mammals that are capable of reducing

Abbreviations: AKR, aldo–keto reductase; SSA, succinic semialdehyde; CBA, carboxybenzaldehyde; IC_{50}, inhibitory concentration of 50% the cells; HNE, 4-hydroxynonenal; MTT, 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide; NQ, naphthoquinone; HGPRT, hypoxanthine guanine phosphoribosyl transferase; DCFH/DA, 2′7′-dichlorodihydrofluorescein diacetate; PQ, phenanthrenequinone; ROS, reactive oxygen species; MUC, *trans*, *trans*-muconaldehyde.

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carbonyls present in a diverse range of natural and synthetic compounds [8]. Several human AKR enzymes have been identified as capable of reducing reactive aldehydes such as 4-HNE, acrolein and methyl glyoxal. These include aldose reductase (AKR1B1) [9,10]; 20α(3α-hydroxysteroid dehydrogenase (AKR1C1) [11]; aldehyde reductase (AKR1A1) [12]; and the aflatoxin B1 aldehyde reductases (AFAR/AKR7A2 and AKR7A3) [13,14]. Of these, the human AKR7A subfamily members are of interest, because they are related to a rat enzyme (AKR7A1) that is inducible by dietary antioxidants [15]. This inducibility has the potential to lead to valuable therapeutic approaches for diseases in which reactive carbonyls have been implicated [5,15].

We have shown previously that mammalian cells expressing rat AKR7A1 are protected from the mutagenicity of acrolein [16]. Rat AKR7A1 has also been shown to be associated with protection against aflatoxin toxicity *in vitro* [15,17], and more recently, using transgenic rats, AKR7A1 has been shown to protect animals against aflatoxin toxicity but not mutagenicity [18,19]. In the mouse, there appears to be only one AKR7A enzyme, AKR7A5, and we have shown previously that V79 cells transfected with mouse AKR7A5 are relatively more resistant to 4-HNE-induced apoptosis [20,21]. Taken together this information suggests that the AKR7A subfamily of enzymes generally serves a cytoprotective function. However little is known of the function of the human members of the AKR7A subfamily. Two human AKR7A enzymes have been isolated, human AKR7A2 and human AKR7A3 [13,14]. Whether the human AKR7A enzymes are similarly involved in detoxication pathways has not been explored previously.

The human AKR7A2 was identified in brain as a succinic semialdehyde reductase [22], and we have shown previously that it is the main enzyme responsible for the reduction of succinic semialdehyde (SSA) to γ-hydroxybutyrate (GHB) in human neuroblastoma cells [23]. AKR7A2 is known to be present in a range of tissues, including liver, kidney as well as brain [12]. However, it appears to accumulate in specific regions of the brain in Alzheimer's Disease [24].

The aim of this study was to test whether the human enzyme AKR7A2 also plays a role in detoxication, as is the case with rat AKR7A1 and mouse AKR7A5. In order to investigate this, we have stably expressed AKR7A2 in V79-4 Chinese hamster lung cells, and have tested the ability of expressed AKR7A2 to protect against the cytotoxicity and mutagenicity of several toxic aldehydes. Furthermore, the role of the AKR7A2 enzyme in enhancing the cell's ability to cope with elevated ROS is also assessed.

2. Methods

2.1. Chemicals

All chemicals were obtained from Sigma except for 4-hydroxynonenal, purchased from Alexis Caymen Corporation – Europe (Nottingham, UK).

2.2. Cell culture

V79-4 Chinese hamster fibroblasts cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle's Media (DMEM) with L-glutamate, 1% (v/v) penicillin and streptomycin, and 5% (v/v) fetal bovine serum.

2.3. Generation V79-4 cell lines stably transfected with AKR7A2

The full length human AKR7A2 cDNA was amplified from an AKR7A2 expression construct [13], a gift from Professor John D. Hayes, and subcloned into the pCI-Neo expression plasmid to give plasmid pCI-Neo-AKR7A2. V79-4 cells were seeded in 24 well plates and pCI-Neo-AKR7A2 was transfected into V79-4 cells using Fugene 6 reagent. Several stable clones were selected as geneticin (G418) resistant. AKR7A2 expression levels in each clone were analyzed by Western blotting. One clone demonstrating strong AKR7A2 expression was maintained for further study.

2.4. Preparation of cell extracts from cell lines

For detection of AKR7A2 expression, whole cell extracts from V79 cells were prepared from 75 cm^2 flasks using a "Freeze–thaw" protocol that does not affect enzyme activity [23]. Briefly, after cells were washed three times in ice-old PBS, 3 ml ice-cold TEN (40 mM Tris–Cl, PH 7.5, 1 mM EDTA, PH 8.0, 150 mM NaCl) solution was added to each flask. Cells were harvested and resuspended in 0.2 M phosphate buffer pH 7.5. The cell suspension was frozen in dry ice for 5 min and then transferred to 37 °C for 5 min. The freeze–thaw procedure was repeated twice. The cell lysate was centrifuged at 12,000*g* for 5 min at 4 °C, and the supernatant was ready for further analysis. Protein concentrations were determined using the method of Bradford against bovine serum albumen standards [25].

2.5. Protein gels and Western blots

Portions of cell extract were separated by SDS–PAGE gel (12%) [26] and transferred onto nitrocellulose membrane (Bio-Rad Inc.). Antisera to AKR7A2 was raised in this lab as previously described [20] and used at a dilution of 1:2000 and goat anti-rabbit IgG-Horse radish peroxidase conjugate secondary antibody (1:10000 dilution). Western blots were developed with enhanced chemiluminescence. (ECL; Amersham) and images were analyzed by LAS-3000 luminescent image analyzer (Fuji). Quantification of band intensities was performed using Kodak one-dimensional image analysis software. A standard curve allowing quantitation of AKR7A5 was achieved by using serial dilutions of purified AKR7A2 recombinant enzyme and plotting concentration versus pixel density of each band determined using NIH Image.

2.6. Expression and purification of recombinant human AKR7A2

Bacterial expression vector pLI19 containing an N-terminal polyhistidine tagged AKR7A2 was a gift from Professor John D. Hayes, University of Dundee [13]. Purification of this enzyme from bacterial cultures was carried out using HiTrap affinity column (5 ml) (Amersham pharmacia biotech) with Pharmacia FPLC (Fast Protein Liquid Chromatography) system. Enzyme activities of purified protein were measured and the most active fractions of protein were combined and frozen at −80 °C until required. Purified protein was also separated by SDS–PAGE to confirm purity.

2.7. Aldo–keto reductase (AKR) assays

Aldehyde- and ketone-reducing activity was determined using an enzyme assay described previously [27], and routinely measured with a Beckman DU650 UV single-beam recording spectrophotometer by following the initial rate of oxidation of NADPH at 340 nm ($\varepsilon_{340} = 6270\ M^{-1}\ cm^{-1}$). The assays were performed at 25 °C in reaction mixtures of 1 ml containing 100 mM sodium phosphate buffer, pH 6.8, and 0.05 mM NADPH. The concentration of the substrate in the reaction mixture was between 10 μM and 10 mM (depending on solubility) with a maximum concentration of 2% (v/v) methanol or 4% (v/v) acetonitrile as carrier; neither methanol nor acetonitrile was found to interfere with the assay or enzyme activity under these conditions. Approximately 1 μg of enzyme or 100 μg cell extract was added to the cuvette to initiate