Chemico-Biological Interactions 191 (2011) 303–307

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

# The role of aldehyde reductase AKR1A1 in the metabolism of gamma-hydroxybutyrate in 1321N1 human astrocytoma cells

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ARTICLE INFO

*Article history:*
Available online 26 January 2011

*Keywords:*
Gamma-hydroxybutyrate
Aldehyde reductase
AKR1A1

ABSTRACT

The role of the aldehyde reductase AKR1A1 in the biosynthesis of gamma-hydroxybutyrate (GHB) has been investigated in cell lines using a specific double stranded siRNA designed to knock down expression of the enzyme. This enzyme, along with the aldo-keto reductase AKR7A2, has been proposed previously to be one of the major succinic semialdehyde reductases in brain. The AKR1A1 siRNA was introduced into the human astrocytoma cell line (1321N1) and AKR1A1 expression was monitored using quantitative reverse-transcriptase PCR and Western blots. Results show an 88% reduction in mRNA levels and a 94% reduction in AKR1A1 protein expression 72 h after transfection with the siRNA. Aldehyde reductase activity was examined in silenced cells by following the aldehyde-dependent conversion of NADPH to NADP at 340 nm. This revealed a 30% decrease in pNBA reductase activity in cell extracts after AKR1A1 silencing. Succinic semialdehyde reductase activity was significantly lower in silenced cells when measured using high concentrations (1 mM) of succinic semialdehyde, but not with low concentrations (10 μM). The effect of silencing on intracellular and extracellular GHB levels was measured using gas chromatography–mass spectrometry. Results show that AKR1A1 has little effect on the production of GHB, indicating that in this cell line alternative enzymes such as the AKR7A2 are likely to play a more significant role in GHB biosynthesis.



## 1. Introduction

γ-Hydroxybutyric acid (GHB) is a small molecule that is synthesized in the brain from γ-aminobutyric acid (GABA) via the intermediate compound succinic semialdehyde (SSA) [1,2]. It is produced through the sequential actions of GABA transaminase [3] and succinic semialdehyde reductase (SSAR) [4]. The biosynthesis of GHB in the brain is of particular interest as several roles have been proposed to account for its presence there. GHB may be involved in maintaining energy balance, and has the ability to lower energy consumption [5]. An endogenous function for GHB in the induction and maintenance of sleep and hibernation has therefore been proposed, in which the consumption of energy is depressed [5]. GHB may also act as a neuroprotectant, protecting cells from anoxia and/or oxidative stress [6]. GHB can also act as a neurotransmitter [7,8], and there are specific G-protein coupled receptors with which it has been shown to interact [9,10].

Previously we have shown that the aflatoxin aldehyde reductase AKR7A2 is one of the major enzymes responsible for GHB biosynthesis in human cells, and acts as a succinic semialdehyde reductase (SSAR), converting SSA to GHB [11,12]. This dimeric enzyme is a member of the aldo-keto reductase superfamily and displays a low $K_M$ for SSA [11]. However, other NADPH-dependent enzymes have also been proposed to play a role in GHB synthesis [13–15]. The presence of high $K_M$ enzymes has not been without controversy and may vary from species to species [16,17]. Several studies have identified cytoplasmic NADPH-dependent SSAR, one of which is classed as the high $K_M$ aldehyde reductase [4], and another low $K_M$ enzyme that is distinct from AKR7A2 but which is highly specific for SSA [4,14,18]. More recently, an iron-dependent alcohol dehydrogenase (ADHFe1), also known as hydroxyacid–oxoacid transhydrogenase (HOT) has been shown to be involved in the oxidation of GHB, but its ability to act as an SSA reductase has not been defined [19,20].

The high $K_M$ SSAR was identified as aldehyde reductase (human AKR1A1; pig AKR1A2; mouse AKR1A4; rat AKR1A3) [18], a member of the aldo-keto reductase superfamily [21]. This enzyme is a monomeric cytosolic oxidoreductase that is expressed in almost all tissues. In the brain of many species, including rat, pig and human, aldehyde reductase is known to be highly expressed, and represents one of the major aldo-keto reductases in this organ [13–15,18].

Aldehyde reductase has been shown previously to have an essential role in ascorbic acid synthesis in mouse [22], but has not

*Abbreviations:* AKR, aldo-keto reductases; GHB, gamma-hydroxybutyrate; SSA, succinic semialdehyde; SSAR, succinic semialdehyde reductase; SSADH, succinic semialdehyde reductase; p-NBA, p-nitrobenzaldehyde.

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doi:10.1016/j.cbi.2011.01.018

been associated with any specific physiological role in humans, despite being widely expressed [23]. Despite progress in understanding the AKR1A1 enzyme, including details of its structure, substrate specificity and expression profile [23–26], a definitive role for this enzyme in GHB biosynthesis has not been previously established. In order to investigate its contribution in more detail, we have used a human astrocytoma cell line 1321N1 [27]. These cells are derived from astrocytes in the brain that perform a range of functions including metabolic support for neuronal cells, and acting as a source for neuroactive substances [28]. Astrocytes are known to metabolize GABA to succinic semialdehyde (SSA) and GHB, and appear to play an important role in endogenous GHB metabolism. They therefore represent a good model for investigating enzymes and pathways of GHB biosynthesis.

In this study, the role of AKR1A1 in the biosynthesis of GHB in 1321N1 human astrocytoma cells has been investigated. Using a specific double stranded siRNA designed to knock down expression of AKR1A1, the effect on SSA reductase activity as well as GHB production is evaluated and the relative contribution of this enzyme is discussed.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals were obtained from Sigma–Aldrich, Poole, Dorset, UK, except for deuterated GHB (GHB-d6), which was obtained from LGC Standards, Teddington, UK.

### 2.2. Antibodies

Antibodies to AKR1A1 were a gift from John Hayes, University of Dundee [23]. Antibodies to GAPDH were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, California.

### 2.3. Cell lines

Human 1321N1 astrocytoma cells [27] were a gift from Dr. Eve Lutz. Cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% of 100 u/ml of penicillin and streptomycin.

### 2.4. RNAi

The siRNA sequence for AKR1A1 (s20198) and a control, scrambled oligonucleotide sequence were obtained from Invitrogen, Paisley, UK. Cells were seeded into 6 well plates ($1 \times 10^6$ cells per well) and transfected with AKR1A1 siRNA at a final concentration of 20 nM using Lipofectamine RNAiMAX transfection reagent (Invitrogen)

### 2.5. Quantitative RT-PCR

Expression of AKR1A1 was assayed by quantitative RT-PCR 72 h after transfection of cells. Total RNA was isolated as described previously [12]. First strand cDNA was synthesized from 5 μg total RNA using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen/Life Technologies). Quantitation of AKR1A1 cDNA was carried out using the Roche LightCycler using AKR1A1 specific oligonucleotide primers (Fwd – 5′-TGCTGCTATCTACGGCAATG-3′; Rev – 5′-TGCATCAGGTACAGGTCCAG-3′). PCRs were carried out as described previously [12] using LightCycler FastStart DNA Master SYBR Green I (Roche). The relative amount of cDNA synthesized in each RT-PCR was compared to GAPDH mRNA levels determined using specific primers.

### 2.6. Preparation of cell extracts

Protein was extracted from 1321N1 cells using a 'freeze–thaw lysis' protocol that does not affect enzyme activity [12]. In brief, cells were washed in phosphate buffer saline (PBS), extracted from 100 mm dishes by scraping and collected by centrifuging. The cell pellet was resuspended in 250 mM Tris buffer pH 7.5. The freeze–thaw procedure was carried out by freezing the cell suspension in −80 °C for 5 min and then thawed at 37 °C for 5 min. This was repeated 3 times and the cell lysate was centrifuged for 5 min at 13,000 rpm and the supernatant was kept in −20 °C. Protein concentrations were determined using the method of Bradford against bovine serum albumen [29]

SDS–PAGE and Western blots – protein samples were separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes as described previously [12]. Membranes were probed for 2 h with primary antibodies (at 1:3000 dilutions) and probed with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG at 1:3000 dilution) for 2 h. Membranes were washed and antibodies detected using enhanced chemiluminescence (ECL; Amersham).

### 2.7. Enzyme assays

Aldo-keto reductase activity was measured by following the change in absorbance of cofactor (NAD(P)(H)) at 340 nm as previously described [12]. All assays were performed at 37 °C in reaction volumes of 1 ml.

### 2.8. Measurement of GHB levels

GHB levels were measured by gas chromatography–mass spectrometry in cells 72 h following siRNA treatment (adapted from [30]) as described previously [12]. Cells were grown in 100 mm dishes. For determining intracellular GHB levels, cells were collected by centrifuging and lysed as described in Section 2.6. For determining extracellular GHB levels, culture media was analyzed. Proteins were precipitated using 500 μl of acetonitrile and were removed by centrifuging for 5 min at 13,000 rpm. The organic layer was transferred to a glass vial and 50 μl of 1 μg/ml of deuterated GHB (GHB-d6) was added to serve as internal standard. The samples were evaporated to dryness under a moderate flow of nitrogen at room temperature and then incubated with 75 μl of the derivative reagent N,O-Bis (Trimethylsilyl) trifluoroacetamide (BSTFA) at 70 °C for 30 min. After cooling to room temperature, 75 μl of ethyl acetate was added and the samples were transferred to a GC–MS vial. 2 μl was injected into a factor four GC–MS column. Ions monitored were 233 for GHB and 239 for GHB-d6.

### 2.9. Statistic analysis

Statistical analysis was performed using GraphPad Prism. Student's *t*-test was used to compare two groups and one way ANOVA was used to compare between more than two groups, with Dunnett's Multiple Comparison post-test.

## 3. Results and discussion

### 3.1. Silencing of AKR1A1

To investigate the role of AKR1A1 in GHB biosynthesis from SSA, human astrocytoma 1321N1 cells were used as they perform a range of metabolic functions and are known to produce neurotransmitters. A doubled-stranded siRNA molecule designed to silence AKR1A1 was transiently transfected into 1321N1 cells in triplicate. 72 h after transfection AKR1A1 mRNA levels were measured by