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Cytotoxicity and chromosomal aberrations induced by acrylamide in V79 cells: Role of glutathione modulators

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ABSTRACT

Acrylamide (AA) is a suspected human carcinogen found to be generated during the heating of carbohydrate-rich foodstuffs. AA exhibits 'Michael-type' reactivity towards reduced glutathione (GSH), resulting *in vivo* in the urinary excretion of mercapturic acid conjugates. GSH is a key factor for mammalian cell homeostasis, with diverse functions that include, among others, the conjugation of electrophilic compounds and the detoxification of products generated by oxidative stress. Therefore, studies focusing on the modulation of GSH are of great importance for the understanding of the mechanisms of AA-induced toxicity. This report addresses this issue by analyzing cytotoxicity (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay) and clastogenicity (chromosomal aberrations) as endpoints in V79 cells after exposure to AA. The experiments described herein include the evaluation of the effect of buthionine sulfoximine (BSO), an effective inhibitor of GSH synthesis, GSH-monoethyl ester (GSH-EE), a compound that is taken up by cells and intracellularly hydrolysed to GSH, and also GSH exogenously added to culture medium. Pre-treatment with BSO increased the cytotoxicity and the frequency of aberrant cells excluding gaps (ACEG) induced by AA. While pre-treatment with GSH-EE did not modify the cytotoxicity or the frequency of ACEG induced by AA, co-treatment with AA and GSH decreased both parameters, rendering the cells less prone to the toxic effects of AA. *In vitro* studies in a cell-free system, using monochlorobimane (MCB), a fluorescent probe for GSH, were also performed in order to evaluate the role of AA in GSH depletion. The results show that spontaneous conjugation of AA with GSH in the extracellular medium is involved in the protection given by GSH. In summary, these results reinforce the role of GSH in the modulation of the cytotoxic and clastogenic effects induced by AA, which may be relevant in an *in vivo* exposure scenario.

1. Introduction

Acrylamide (AA) is a suspected human carcinogen generated during the heating of carbohydrate-rich foodstuffs, predominantly from the precursor asparagine [1]. Acrylamide toxicity to mammalian cells has been described and characterized in the last few years using different approaches [2,3].

AA is metabolized to glycidamide (GA) by an epoxidation reaction, presumably mediated by cytochrome P450 2E1 (CYP2E1) [4,5]. This metabolic conversion appears to be critical for the genotoxicity of AA [2,3,6,7]. Recently, we have also found that AA, compared to GA, is clearly less cytotoxic and genotoxic, as evaluated by different endpoints [8]. In fact, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay or the DNA damage endpoints studied, *viz* chromosomal aberrations (CAs), sister chromatid exchange (SCE) and glycidamide–DNA adducts (GA–DNA adducts), showed that GA unequivocally possesses more toxicity than AA. However, while the importance of GA is nowadays convincing, further studies focusing on the metabolic fate of AA are still needed for a better mechanistic understanding of its toxicity. The modulation of reduced glutathione (GSH) status could give additional insight into this matter. GSH has several crucial roles in mammalian cells including, among others, the conjugation of electrophilic compounds and the detoxification of products generated by oxidative stress [9]. Both GSH functions can indeed be important for mitigation of AA toxicity in an *in vivo* exposure scenario. In fact, besides the possibility of AA being an oxidative stress inducer [10–13], this compound is efficiently conjugated with reduced glutathione (GSH) [3,14]. The α,β-unsaturated carbonyl group of AA allows its 'Michael-type' reactivity toward

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GSH, resulting in the urinary excretion of mercapturic acid conjugates [3]. The conjugates of GSH with AA have been quantified in humans and their importance reported. In fact, toxicokinetic studies in humans have shown that ~60% of AA can be recovered in the urine [15], essentially in the form of GSH conjugates [16]. GA is also conjugated with GSH, but the ratio of glycidamide–GSH to acrylamide–GSH conjugates excreted in human urine is only ~0.1 [15].

In this report, we describe the evaluation of the effect of the GSH modulators buthionine sulfoximine (BSO), a specific inhibitor of GSH synthesis, and GSH-monoethyl ester (GSH-EE), a compound that is taken up by cells and intracellularly hydrolysed to GSH. While BSO pre-treatment effectively reduces the endogenous content in GSH [17], the pre-loading of mammalian cells with GSH-EE has proven to significantly increase GSH intracellular content [18]. In this work, we performed cell viability studies using the MTT reduction assay and also evaluated the levels of chromosomal damage by use of the chromosomal aberration assay in V79 Chinese hamster fibroblasts. In addition, the simultaneous treatment of AA with exogenously added GSH was studied using the same endpoints. In this co-treatment protocol the effect of GSH is essentially extracellular, since this compound does not easily enter the cells [19]. In view of this, we have performed *in vitro* studies in a cell-free system, using monochlorobimane (MCB), a fluorescent probe for GSH [20], in order to evaluate the depletion of GSH in the presence of AA and thus the role of the spontaneous, non-enzymatic conjugation of AA with GSH.

2. Materials and methods

2.1. Chemicals

L-Glutathione reduced (GSH; CAS registry number 70-18-8), glutathione reduced monoethyl ester (GSH-EE; CAS registry number 92614-59-0), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), trypsin, newborn calf serum, Ham's F-10 medium, phosphate-buffered saline pH 7.4 (PBS), mitomycin C, and penicillin–streptomycin solution were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), ethanol, methanol, acetic acid, potassium chloride, sodium chloride, and Giemsa dye were obtained from Merck (Darmstadt, Germany). Colchicine, monochlorobimane (MCB), L-buthionine sulphoximine (BSO; CAS registry number 83730-53-4), and acrylamide (AA; CAS registry number 79-06-1, ≥99.5% pure) were purchased from Fluka (Buchs, Switzerland).

2.2. MTT reduction assay

The MTT assay was performed according to Martins et al. [8] with V79 Chinese hamster fibroblasts (MZ), kindly provided by Prof. H. Glatt (German Institute of Human Nutrition, Germany). The MTT assay is based on the reduction of the yellow MTT tetrazolium salt by mitochondrial dehydrogenases to form a blue MTT formazan, in viable cells [21,22].

Ten-hour cultures, growing in 96-well micro-plates were incubated with BSO (0.1 mM) or GSH-EE (1.0 and 2.5 mM) for a period of 14 h. Afterwards the medium was removed, the cells were washed with culture medium, and exposed to AA (2.0 and 4.0 mM) for 24 h. In the co-incubation experiments, cells exposed to AA (0.5–6.0 mM) were simultaneously incubated with GSH (1.0 mM). After 24 h of incubation the cells were washed and MTT (0.5 mg/ml) was added to each well. The cells were grown for a further period of 3 h and then carefully washed with PBS. DMSO (200 μl) was added to each well and absorbance values were read in an Anthos Zenyth 3100 multimode detection micro-plate reader at 595 nm. Absorbance values presented by V79 cell cultures without the addition of AA, BSO, GSH-EE or GSH, i.e. control cultures, correspond to 100% of cell viability. At least two independent experiments were performed. Eight individual cultures were used in each independent experiment.

2.3. Chromosomal aberration assay

The chromosomal aberration assay was performed as described previously [8,23]. V79 cells (24-h cultures) were exposed to 2.0 mM AA for a period of 16 h. BSO (0.1 mM) and GSH-EE (1.0 mM) were added 14 h before the incubation with AA. In the co-incubation experiments, cells were simultaneously exposed to AA and GSH (1.0 and 3.0 mM). Mitomycin C (0.75 μM) was used as the positive control. After the treatment with AA, the cells were washed with fresh culture medium and colchicine was added at a final concentration of 0.6 μg/ml. The cells were incubated for a further 2.5 h and then harvested by trypsinization. After a 3-min hypotonic treatment with KCl (0.56%, w/v) at 37 °C, the cells were fixed with methanol/acetic acid (3:1), and slides were stained with Giemsa 4% (v/v) in phosphate buffer 0.01 M, pH 6.8 for 10 min. Two independent experiments were performed, except for the co-treatment of AA with 1.0 mM GSH. In this case four independent experiments were performed.

For each independent experiment, 100 well-spread metaphases were observed with a 1250× magnification on a light microscope. Scoring of the different types of aberration was performed according to published criteria [24,25]. For the quantification of the DNA damage induced by AA, the index %ACEG (percent of aberrant cells excluding gaps) was used. This index represents the frequency of metaphases that contain chromosomal aberrations excluding gaps and constitutes the standard indicator for the chromosomal aberration assay. The types of aberration considered for this index were breaks (chromatid and chromosome), dicentric chromosomes and rings, chromatid-type rearrangements (triradial, quadriradial), other complex rearrangements and multi-aberrant cells (MA, cells with more than 10 aberrations, including heavily damaged pulverized cells).

2.4. GSH conjugation assay

The conjugation studies of AA with GSH were carried out in 96-well black microplates using an *in vitro* cell-free assay based on the MCB fluorimetric method [20,26]. MCB is a probe that reacts with GSH generating an adduct (MCB–GSH conjugate) that can be detected by fluorimetry [20].

To each well was first added PBS, and then AA (up to 6.0 mM final concentration) and/or GSH (up to 1.0 mM final concentration). The micro-plates were placed in an incubator at 37 °C, protected from light for a period of 1 or 24 h. Afterwards, MCB solution (stock solution of 10 mM in absolute ethanol, diluted 10× with PBS) was added to each well at a final concentration of 100 μM. Control experiments were performed with AA and GSH without any incubation. The plates were then placed in an incubator with shaker for 30 min at 37 °C (100 rpm, protected from light) and fluorescence was measured in an Anthos Zenyth 3100 multimode detection microplate reader (λ_{exc} = 405 nm, λ_{em} = 465 nm).

The results were expressed either in fluorescence intensity using relative fluorescence units (RFU) after subtraction of the background fluorescence, or normalized in terms of free GSH (%), i.e. the mean value of fluorescence (RFU) observed in GSH alone plus MCB wells corresponded to 100%. For each experimental point four wells were used in each independent experiment. At least two independent experiments were carried out.

2.5. Statistical analysis

The Kolmogorov–Smirnov test was used to test the normality of continuous variables (% cell viability, %ACEG). For the variables with a normal distribution the homogeneity of the variances was evaluated using the Levene test, and the differences in the mean values of the results observed in cultures treated with AA *versus* AA + GSH modulators were evaluated by Student's *t*-test. For non-normal variables the Mann–Whitney was used. The levels of significance considered were $P < 0.05$ and $P < 0.01$. All analyses were performed with the SPSS statistical package (version 15, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. MTT reduction assay

The results from a set of cell-based experiments using the MTT reduction assay are presented in Fig. 1. These experiments were performed in order to evaluate the effect of GSH modulators on the viability of V79 cells treated with AA.

Fig. 1A presents the effect of the depletion of intracellular GSH by pre-incubation of V79 cells with BSO. BSO alone slightly reduced cell viability compared with that of control cells (Fig. 1A). The viability of AA-treated V79 cells decreased as a function of AA concentration (Fig. 1A). The pre-incubation with 0.1 mM BSO caused an additional decrease in viability of 35% at 4 mM of AA ($P < 0.05$).

The effect of the pre-treatment with GSH-EE on the viability of V79 cells exposed to AA is shown in Fig. 1B. In these experiments, two concentrations of GSH-EE were used (1.0 and 2.5 mM). GSH-EE alone at 1.0 mM concentration was not cytotoxic, but the high concentration (2.5 mM) caused a decrease in cell viability of about 17%. The cell viability in AA-treated cultures was not significantly altered in the presence of 1.0 or 2.5 mM GSH-EE.

Cell viability data corresponding to simultaneous treatment of V79 cells with AA and GSH are presented in Fig. 1C. A slight decrease in cell viability was observed for GSH alone (1.0 mM). Co-treatment