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Comparison of glutathione levels measured using optimized monochlorobimane assay with those from ortho-phthalaldehyde assay in intact cells

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ABSTRACT

Fluorometric glutathione assays have been generally preferred for their high specificity and sensitivity. An additional advantage offered by fluorescent bimane dyes is their ability to penetrate inside the cell. Their ability to react with glutathione within intact cells is frequently useful in flow cytometry and microscopy. Hence, the aims of our study were to use monochlorobimane for optimizing a spectrofluorometric glutathione assay in cells and then to compare that assay with the frequently used *ortho*-phthalaldehyde assay. We used glutathione-depleting agents (e.g., cisplatin and diethylmalonate) to induce cell impairment. For glutathione assessment, monochlorobimane (40 μM) was added to cells and fluorescence was detected at 394/490 nm. In addition to the regularly used calculation of glutathione levels from fluorescence change after 60 min, we used an optimized calculation from the linear part of the fluorescence curve after 10 min of measurement. We found that 10 min treatment of cells with monochlorobimane is sufficient for evaluating cellular glutathione concentration and provides results entirely comparable with those from the standard *ortho*-phthalaldehyde assay. In contrast, the results obtained by the standardly used evaluation after 60 min of monochlorobimane treatment provided higher glutathione values. We conclude that measuring glutathione using monochlorobimane with the here-described optimized evaluation of fluorescence signal could be a simple and useful method for routine and rapid assessment of glutathione within intact cells in large numbers of samples.

1. Introduction

Glutathione, γ-L-glutamyl-L-cysteinylglycine, is a low molecular weight thiol occurring in two free forms – the reduced form (GSH) and glutathione disulfide. It is a crucial intracellular antioxidant due its role in detoxification of free radicals, regeneration of other antioxidants, and maintaining of SH-groups in intracellular proteins. Additional roles of glutathione involve detoxification of xenobiotics, regulation of redox environment in the cell, and transport of cysteine (Dringen 2000; Forman et al. 2009; Sies 1999). All these functions point to this molecule's significance. Therefore, knowledge of glutathione levels in cells is of crucial importance, and especially so in cell toxicity studies.

A number of assays based on a variety of analytical procedures have been used for glutathione detection. The most used are liquid chromatography methods. Their advantages lie in their high specificity and their sensitivity, the latter depending on the type of detection used, be that UV (Amarnath et al. 2003; Garcia et al. 2008; Glowacki and Bald 2009), mass spectrometry (Dieckhaus et al. 2005; Dirven et al. 1994; Guan et al. 2003; Paroni et al. 1995), or electrochemical (Hiraku et al. 2002; Kominkova et al. 2015). Their main disadvantages are their costliness and long time required for their analytical processes. The other glutathione assay most often used is a spectrophotometric method. Here, the glutathione reacts with 5,5′-dithiobis-2-nitrobenzoic acid (Ellman 1959; Eyer and Podhradsky 1986). This method has been known also as the recycling assay (Tietze 1969). The advantages of the recycling assay are its great sensitivity and short measurement time. Its disadvantage can be seen in possible interference of glutathione reductase inhibiting compounds (Griffith 1980). Other methods for glutathione measurement are based on bioluminescence (Romero and Mueller-Klieser 1998), electrochemistry (Inoue and Kirchhoff 2000; Safavi et al. 2009), gas chromatography (Kataoka et al. 1995), and capillary electrophoresis (Oshea and Lunte 1993; Shackman and Ross 2007).

The latter group of methods for glutathione analysis consists of fluorometric assays. These methods are often preferred for their high sensitivity and specificity. The probe most used, *ortho*-phthalaldehyde

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(OPA), can react with GSH to form a highly fluorescent product (Cohn and Lyle 1966; Hissin and Hilf 1976; Rousar et al. 2012). The analogue of OPA, 2,3-naphthalenedicarboxaldehyde, has been used for glutathione detection, too (White et al. 2003). The ratiometric probe ThiolQuant Green has been developed for intracellular GSH quantitation recently (Jiang et al. 2015). Still other fluorescent probes, bimanes, have been used less frequently.

Presently, the bimanes monochlorobimane (MCB) and monobromobimane (MBB) can be used for detecting GSH and other thiols (Anderson et al. 1999; Barhoumi et al. 1995; Chatterjee et al. 1999; Fernandezcheca and Kaplowitz 1990; Kamencic et al. 2000; Kosower and Kosower 1987; Kosower et al. 1979). In comparison to OPA, their substantial advantage can be seen in their ability to penetrate into the cell through cell membrane and to react directly with cellular thiols. This ability has been utilized in fluorescence microscopy assessment (Bellomo et al. 1992; Keelan et al. 2001; Stevenson et al. 2002) and flow cytometry (Han et al. 2016; King et al. 2004; Xiao et al. 2016). On the other hand, quantitative spectrofluorometric assessment of glutathione levels in intact cells has not yet been used frequently. Because a method for GSH assessment using bimanes could be rapid, sensitive, and specific, and therefore suitable for routine assessment of GSH in intact cells, the aim of the present study was to optimize the bimanes-derived glutathione assay for this purpose. In addition, there is no comparison in the literature of bimanes-derived GSH assay with any other glutathione assay. An additional aim of our study, therefore, was to compare glutathione results obtained using our optimized bimane-derived method with those from the standard *ortho*-phthalaldehyde-derived method.

2. Materials and methods

2.1. Chemicals

All chemicals (OPA, MCB, MBB, diethylmalonate, cisplatin, $HgCl_2$, H_2O_2, glutathione, and *tert*-butyl hydroperoxide) were purchased from Sigma–Aldrich (USA).

2.2. Cell culture

Two cell lines (HK-2 and A549) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human kidney (HK-2) cells are a proximal tubular epithelial cell line derived from normal adult human kidney cells immortalized by transduction with human papillomavirus (HPV 16) DNA fragment (Ryan et al. 1994). The cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12 = 1:1) supplemented with 5% (v/v) fetal bovine serum, 1 mM pyruvate, 50 μg/mL penicillin, 50 μg/mL streptomycin, 10 μg/mL insulin, 5.5 μg/mL transferrin, 5 ng/mL sodium selenite, and 5 ng/mL epidermal growth factor and maintained at 37 °C in a sterile, humidified atmosphere of 5% CO_2.

The adenocarcinomic human alveolar basal epithelial cell line (A549) is derived from human lung adenocarcinoma cells (Foster et al. 1998). The cells were cultured in Minimum Essential Medium Alpha (MEM α) without nucleosides supplemented with 10% (v/v) fetal bovine serum, 1 mM pyruvate, 10 mM HEPES, and 50 μM/ml penicillin/streptomycin and maintained at 37 °C in a sterile humidified atmosphere of 5% CO_2.

2.3. Measurement of glutathione levels – bimanes assay

The working solutions of MBB and MCB were prepared fresh at the time of analysis by dilution in Dulbecco's phosphate buffer (pH 7; 1 mM) and tempered at 37 °C. The cells were incubated in cell medium (100 μL) on 96-well plates with the tested compounds for appropriate time periods: diethylmalonate (DEM; 0.5 and 2 mM; 3 h), *tert*-butyl hydroperoxide (50 and 500 μM; 24 h), cisplatin (5, 30 and 50 μM; 24 h), and $HgCl_2$ (20 μM; μM; 24 h). After incubation, 20 μL of the bimane solution was added to cells and the measurement was started. The final concentrations of MCB and MBB in a well were 40 μM and 10 μM, respectively. The fluorescence (Ex/Em = 394/490 nm) was measured for 60 min using a Tecan Infinite M200 fluorescence reader (Tecan, Austria) while incubated at 37 °C. We compared two calculations of glutathione levels from the fluorescence signal: 1) "standard" – the fluorescence was calculated as the difference between the fluorescence signal at 60 min and 0 min; and 2) "optimized" – the fluorescence was expressed as the slope of change of fluorescence/time on a linear part of the curve after 10 min of measurement (Δ fluorescence$_{(0–10\ min)}$/10 min). The glutathione levels were expressed as the percentage relative to glutathione levels in control cells (control = 100%).

2.4. Measurement of glutathione levels – ortho-phthalaldehyde assay

The measurement of glutathione levels using OPA was performed according to the published protocol (Rousar et al. 2012). The cells were lysed in cold 10% metaphosphoric acid for 10 min at 4 °C. The cells were then scraped and cell lysates were centrifuged (5 min, 4 °C, 20.000 *g*). The supernatant was used for the fluorometric assay. Briefly, 50 μL of supernatant was mixed with 1000 μL of phosphate buffer (100 mM; ph 8; with 1 mM EDTA), then 60 μL of the mixture was again added to 900 μL of phosphate buffer and 60 μL of OPA (0.1%; w/v in methanol). After 15 min, 75 μL of hydrochloric acid (1 mM) was added and the samples and standards were incubated at 4 °C until analyzed. The fluorescence was measured at Ex/Em = 340/420 nm using an Aminco Bowman 2 spectrofluorometer (Thermo, USA).

2.5. Comparison of bimanes and ortho-phthalaldehyde assays

To compare the use of MCB and MBB to detect glutathione levels in cells, we used the reference fluorometric method based on reaction of glutathione with OPA. All three methods were used for detecting glutathione levels in the two cell lines (HK-2, A549) treated with the glutathione-depleting agent DEM (0.5 and 2 mM). After 3 h incubation, the glutathione concentrations were measured and calculated as percentages of levels in untreated control cells (control = 100%).

An additional comparison was performed between the MCB and OPA assays. The two cell lines (HK-2, A549) were treated with selected toxic compounds. We used DEM (0.5 and 2 mM), *tert*-butyl hydroperoxide (50 and 500 μM), cisplatin (5, 30 and 50 μM), $HgCl_2$ (20 μM), and hydrogen peroxide (500 μM). The toxic compounds were diluted in cell culture medium and the cells were treated for 24 h. The results were used to calculate the correlation coefficient for the MCB-derived method versus the reference method using OPA ($n = 20$).

2.6. Statistical analysis

All experiments were repeated at least three times independently. All values were measured at least in duplicate. The data were expressed as glutathione levels as a percentage of control values (control = 100%). The results were expressed as mean ± SD or mean ± SEM. The differences between groups of samples measured by different assay were evaluated using one-way analysis of variance (ANOVA), $p = 0.05$ (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). The correlation coefficient between OPA and MCB assays was calculated after analysis of similar samples.

3. Results

3.1. Optimized calculation of GSH levels detected using MCB

Monochlorobimane has been used for detection of cellular glutathione mostly in flow cytometry and fluorescence microscopy (Barhoumi et al. 1995; Bellomo et al. 1992; Chan and Kwong 2000;

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