RESEARCH COMMUNICATION

Distribution of the monochlorobimane–glutathione conjugate between nucleus and cytosol in isolated hepatocytes

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The nuclear/cytoplasmic distributions of the GSH and *N*-acetylcysteine conjugates of monochlorobimane (MCB) have been studied in rat hepatocytes in primary culture after microinjection into the cytoplasm. The immediately occurring fluorescence (within 1–2 s) in the nucleus was much greater than that in the cytoplasm. Other water-soluble fluorescent markers such as Cascade Blue, 6-carboxyfluorescein and Lucifer Yellow also exhibited a similar nuclear localization. MCB, which is poorly soluble in water, gave similar results whether exposed to the

exterior of the cell or microinjected as a solution in ethanol. It is concluded that the fluorophores studied here diffuse into the nucleus and accumulate there, including MCB–GSH formed intracellularly from MCB and GSH. Therefore MCB may be of limited value in estimating concentrations of GSH in the nucleus and the cytoplasm, as recently suggested by Bellomo, Vairetti, Stivala, Mirabelli, Richelmi and Orrenius [(1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4412–4416].

INTRODUCTION

In order to protect the nuclear structures from damage, glutathione must permeate the nuclear envelope. Values for the nuclear/cytoplasmic distribution of GSH vary according to the technique of estimation used. Subcellular fractionation of rat liver by aqueous techniques gives nuclei which, compared with the soluble supernatant fraction, contain little or no GSH (Blobel and Potter, 1966; Taylor et al., 1973), but fractionations of rat kidney (Tirmenstein and Reed, 1988) or liver (S. Soboll, S. Gründel, J. Harris, H. Sies and B. Ketterer, unpublished work) by using non-aqueous conditions give similar GSH values in both cytoplasm and nucleus. It is assumed that during aqueous, but not non-aqueous, fractionations nuclear GSH is lost through the nuclear pores. Recently, an interesting study intended to measure intracellular GSH in the unruptured cell was carried out (Bellomo et al., 1992). Intact hepatocytes in culture were incubated with monochlorobimane (MCB), which permeates cells and gives a strongly fluorescent glutathione conjugate (MCB–GSH) due to the catalytic action of intracellular GSH S-transferases (GSTs) (Hulbert and Yakubu, 1983). Fluorescence was preferentially localized in the nucleus, in apparent accord with a nuclear/cytoplasmic GSH gradient of about 3-fold. Although Bellomo et al. (1992) give some evidence that the nuclear/cytoplasmic concentration gradient of GSH is maintained by an active mechanism, the possibility also exists that MCB–GSH formed anywhere in the cell diffuses freely through nuclear pores to reach physicochemical equilibrium between the nucleus and the cytoplasm. If this is the case, the observed gradient may be dependent on factors such as differences between the two cellular compartments with respect to free water volume, binding interactions involving MCB–GSH, a longer light path through the nucleus, fluorescence yield etc.

We have investigated the subcellular distribution of MCB–GSH and the *N*-acetylcysteine conjugate of MCB (MCB–NAC),

and also other fluorescent dyes used in cell culture, in rat hepatocytes after microinjection into the cytoplasm. All these probes gave immediate prominent fluorescence in the nucleus.

MATERIALS AND METHODS

MCB, Cascade Blue and 6-carboxyfluorescein were obtained from Molecular Probes (Eugene, OR, U.S.A.); GSH and NAC were from Boehringer (Mannheim, Germany); Lucifer Yellow was from Sigma (Deisenhofen, Germany); cell culture medium and gentamycin were from Gibco (Eggenstein, Germany). To obtain conjugates, MCB (3 mg) was reacted with GSH or NAC (1.5–2 equivalents) in 100 mM sodium phosphate (pH 8.0)/ethanol (1:1, v/v) overnight, and ethanol was then removed by evaporation at 50 °C. The resulting aqueous solution was then applied to a Sep-Pak cartridge (Milford, U.K.) and GSH–MCB or NAC–MCB was eluted with methanol/water (1:3, v/v). The purity of conjugates was checked by cellulose t.l.c., with the solvent butan-1-ol/acetic acid/pyridine/water (15:3:10:12, by vol.). Hepatocytes were isolated from male Wistar rats (250 g) by EDTA perfusion of the liver (Wright and Paine, 1992) and were placed in 35 mm × 10 mm collagen-coated Petri dishes at 200 000 cells/ml in Williams' Medium E containing 10% (w/v) foetal-calf serum and 40 µg/ml gentamycin. Cells were cultured at 37 °C in an atmosphere of 5% CO₂ in air for 24 h before microinjection of fluorochromes.

MCB–GSH (40 mM), MCB–NAC (50 mM), Cascade Blue (1 mM) and 6-carboxyfluorescein (1 mM) in 50 mM Hepes, pH 7.4, and MCB (20 mM) in ethanol, were injected into the cytoplasm of cells with the aid of a Microinjector 5242 and ECET Micromanipulator 5170 (Eppendorf, Hamburg, Germany). For microinjection a pressure of 20 kPa was applied for 0.1 s. From hepatocellular dimensions derived by Drabkin (1975), it can be calculated that the injected material is diluted approx. 150-fold by the volume of the cell. Fluorescent images were

Figure 1 Fluorescent images of the subcellular distribution of GSH-MCB (a), NAC-MCB (b), MCB (c), Cascade Blue (d), 6-carboxyfluorescein (e) and Lucifer Yellow (f) in rat hepatocytes immediately (i.e. 3–10 s) after microinjection of fluorescent markers into the cytoplasm

obtained with an IM 35 fluorescence microscope (Zeiss, Oberkochen, Germany).

Kinetic constants for the catalysis of MCB conjugation by the major GSTs present in the liver were determined by using purified enzymes and the spectrofluorimetric determination of MCB-GSH concentration using excitation at 392 nm and emission at 482 nm (Fraser, 1992).

RESULTS AND DISCUSSION

The fluorescence image of hepatocytes after microinjection of MCB is shown in Figure 1. Development of fluorescence is rapid, due to the GST-catalysed conjugation of MCB with intracellular GSH. Previously estimated concentrations of GST isoenzymes in isolated hepatocytes (Vandenbergh et al., 1988) and kinetic constants determined for purified isoenzymes (Table 1) indicate that the rate of formation of MCB-GSH is approx. 140 $\mu\text{M/s}$. Therefore, microinjected MCB should be rapidly converted into the conjugate in all compartments of the cell containing GSH and GST. Under the conditions of this experiment, fluorescence

accumulates in the nucleus within 1–2 s. Although Bellomo et al. (1992) have interpreted similar images obtained upon incubation of cells with MCB to mean that the nucleus contains a higher concentration of GSH than does the cytoplasm, it is shown in Figure 1 that incubation with MCB and microinjection with MCB-GSH give similar distributions of fluorescence. This shows that the nuclear/cytoplasmic gradient of MCB-GSH observed after exposure to MCB is not necessarily due to a higher concentration of GSH in the nucleus. Furthermore, microinjection of MCB-NAC gave the same result (Figure 1b), showing that the presence of a greater fluorescence due to MCB-GSH in the nucleus compared with that in the cytoplasm does not depend on the presence of the GSH moiety in the fluorescent compound. Finally, the bimeane moiety itself is not essential for nuclear accumulation, since the chemically unrelated water-soluble fluorescent dyes Cascade Blue, 6-carboxyfluorescein and Lucifer Yellow on microinjection give an intracellular distribution similar to that of the bimeane derivatives (Figures 1d–1f).

These results show that MCB may be of limited value in estimating concentrations of GSH in the cytoplasm and the nucleus, particularly since the rate of diffusion of small molecules is such that MCB-GSH should traverse the hepatocyte in approx. 1 s (Setlow and Pollard, 1962).

Table 1 Kinetic constants for the catalysis of GSH conjugation of MCB by the major GSTs of rat liver

For details, see Fraser (1992).

GST isoenzyme	K_m (μM)	V_{max} ($\mu\text{mol/min per mg}$)
1-1	13.3 ± 3.5	0.26 ± 0.03
2-2	20.7 ± 4.2	0.06 ± 0.01
3-3	0.09 ± 0.02	0.55 ± 0.02
4-4	19.5 ± 1.85	1.55 ± 0.07

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