Review Article

Glutathione Regeneration in Mammalian Erythrocytes

M. Kurata¹, M. Suzuki² and N. S. Agar³

¹Drug Safety Evaluation, Pfizer Pharmaceutical Co. Ltd, Aichi; ²The Faculty of Veterinary Science, Gifu University, Japan; ³Department of Physiology, The University of New England, Armidale, Australia

Abstract. Glutathione (GSH) regeneration is a process in which cells reduce oxidised glutathione (GSSG) to GSH after exposure of cells to oxidants in the presence of suitable energy source such as glucose. This reaction consists of (1) membrane glucose transport, (2) phosphorylation of glucose by hexokinase (HK) utilising adenosine 5’-triphosphate (ATP), (3) reduction of nicotinamide–adenine dinucleotide phosphate (NADP) to its reduced form (NADPH) by glucose-6-phosphate dehydrogenase (G-6-PD) and 6-phosphogluconate dehydrogenase (6-PGD) and (4) reduction of GSSG to GSH by glutathione reductase (GR) using NADPH. The rate of GSH regeneration is thus dependent on the enzymatic activity and concentration of substrate. G-6-PD deficiency and enzyme inhibitory chemicals reduce GSH regeneration and concentration of substrate greatly influences the rate of GSH regeneration. Not only glucose but also mannose, fructose and galactose may also be used as energy source. Interspecies and intraspecies differences occur in the rate of GSH regeneration. These differences cannot be explained by variations in enzymatic activities. Although physiological relevance of GSH regeneration is still unclear, it is known that erythrocytes from G-6-PD-deficient patients have lowered erythrocyte deformability and shortened erythrocyte life-span. In-vitro experiments show lowered GSH regeneration enforces loss of deformability. These findings suggest that GSH regeneration plays an important role in erythrocyte biology.

Keywords: Erythrocyte; Glutathione; Mammal; Redox cycle; Regeneration

Introduction

Glutathione (GSH) (γ-glutamyl-cysteinyl-glycine) is the most abundant compound of low molecular weight present in mammalian cells including erythrocytes. One of the major functions of GSH in cells is to reduce harmful oxidants by converting itself into an oxidised form (GSSG). GSH is then regenerated from GSSG. This process called ‘GSH regeneration’, consists of four steps: (1) glucose transport through the erythrocyte membranes; (2) phosphorylation of glucose to glucose-6-phosphate (G-6-P) by hexokinase (HK); (3) reduction of nicotinamide–adenine dinucleotide phosphate (NADP) to its reduced form (NADPH) by glucose-6-phosphate dehydrogenase (G-6-PD) and 6-phosphogluconate dehydrogenase (6-PGD) and finally (4) reduction of GSSG to GSH by glutathione reductase (GR) utilising NADPH.

Kosower et al. (1967) first introduced GSH regeneration as a screening test for human erythrocyte G-6-PD deficiency. Since then the test has been extensively used in human and veterinary medicine using several substrates and oxidising agents. Inter- and intraspecies differences in GSH regeneration have been reported in several mammalian erythrocytes. The aim of the present paper is to integrate this widely scattered information in one place so that future workers may appreciate the origin and significance of GSH regeneration in erythrocyte biology, factors affecting the rate of GSH regeneration, efficacy of different substrates and oxidising agents and overall physiological role of this important and commonly used biological tool.

Methodology

Erythrocytes are usually obtained from human and/or animals and anticoagulated with heparin or ethylenedia-
minetetraacetic acid (EDTA) then washed with an appropriate buffer or saline. The cells are suspended in an isotonic buffer, e.g. glycyl-glycine buffer (Kosower et al. 1967), phosphate buffer (Frischer and Ahmad 1987) or Hepes buffer (Kurata and Suzuki 1994). An oxidant is added to the erythrocyte suspension followed by an appropriate substrate. Aliquots are taken at different time intervals for GSH regeneration.

The test is carried out immediately after collecting blood, because if the erythrocytes are left more than a few hours in whole blood or buffer there is a decrease in intrinsic glucose, ATP and other metabolites. Initiation of the regeneration process is delayed as a result of these events (Suzuki and Kurata 1992; Bozzi et al. 1996).

Though GSH regeneration is usually carried out using intact cells, it can also be done in a mixture of substrates and purified enzymes. Thorburn and Kuchel (1985) employed such a system. Dialysed haemolysate in combination with glucose, ATP and NADPH may also be used (Kurata and Suzuki 1994).

**Oxidants**

Choice of oxidant is an essential part in the process of GSH regeneration. Furthermore, the amount of oxidant used is also an important consideration. Slight excess of oxidant causes a delay in GSH regeneration (Kosower et al. 1969). Oxidants that have been used in the test for GSH regeneration are as follows.

**Methyl Phenylazoformate**

Methyl phenylazoformate (azoester) was the first oxidant employed in GSH regeneration studies by Kosower et al. (1965). Several subsequent studies also used this oxidant (Kosower et al. 1967; Smith and Parks 1968; Rieber and Jaffe 1970). Azoester oxidises approximately 30% of haemoglobin to methaemoglobin, and is not specific for GSH (Kosower et al. 1965; Rieber and Jaffe 1970).

**Diamide**

Kosower et al. (1969) introduced deizenedicarboxylic acid bis(N,N-dimethylamide; diamide) as a substitute for azoester. Diamide has the ability to oxidise GSH to GSSG non-enzymatically (Sies 1985) and is more specific for oxidation of GSH than azoester. This reagent has been commonly used in GSH regeneration (see Table 1).

Diamide, though relatively specific for GSH, causes oxidative damage in cells at higher concentrations. Harris and Biaglow (1972) reported that in tumour cell lines this reagent reacts with 15–20% of protein-bound-SH groups and is not absolutely specific for GSH. In erythrocytes, diamide produces disulphide bonds in membrane proteins, resulting in loss of erythrocyte deformability (Frischer et al. 1978; Haest et al. 1980; Maeda et al. 1983; Kurata et al. 1994).

**Acetylphenylhydrazine**

Acetylphenylhydrazine (APH) is a classical oxidant and has been employed for the ‘GSH stability test’ (Beutler 1957; Agar et al. 1975). Kosower et al. (1967) noted that APH is unsuitable for studying GSH regeneration because the presence of excess active reagent results in loss of GSH regeneration rate. APH forms significant amounts of haemoglobin–GSH mixed disulphide (Srivastava and Beutler 1969), and produces Heinz-bodies in erythrocytes (Beutler 1957; Goto et al. 1991). Frischer and Ahmad (1987), nevertheless, employed APH as an oxidant for GSH regeneration.

**Hydrogen Peroxide**

Hydrogen peroxide (H₂O₂) is a physiological oxidant mainly derived from superoxide radical via an enzymatic reaction of superoxide dismutase. This agent is also catalysed by catalase and thus is not specific for oxidation of GSH. Cohen and Hochstein (1961) used H₂O₂ as an oxidant for GSH stability test.

**t-Butyl Hydroperoxide**

t-Butyl hydroperoxide (tBH) acts as a substrate for GSH-Px which converts GSH into GSSG. Unlike H₂O₂, this peroxide is not catalysed by catalase. Sies and Gerstenecker (1972) used this oxidant in studying GSH metabolism in perfused liver. Srivastava et al. (1974) demonstrated that tBH may be a selective oxidant for GSH in erythrocytes. Mahaffey and Smith (1975) also employed this agent in GSH regeneration in erythrocytes.

GSH is regenerated more rapidly with tBH than with diamide (Suzuki et al. 1983). Accordingly, tBH is now the most standard oxidant used in GSH regeneration studies (Table 1). However, tBH at high concentration, like other oxidants, produces oxidative damage (e.g. lipid peroxidation and protein degradation) at high concentration via production of reactive oxygen species (Corry et al. 1980; Trotta et al. 1982, 1983).

**Hypochlorous Acid**

Hypochlorous acid (HOCl) can penetrate erythrocyte membrane and oxidise GSH in a highly selective process. By exposing this, virtually all of the GSH lost is converted into GSSG and rapidly regenerated in the presence of glucose (Visser and Winterbourn 1995).