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Haemoglobin Oxidation in Whole Blood Samples from Sheep in relation to Glutathione Peroxidase Activity

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Abstract

A high correlation was observed between H_2O_2-induced oxidation of haemoglobin and glutathione peroxidase activity in whole blood samples from sheep. A role of this enzyme in the prevention of oxidative damage to the erythrocyte and its contents has been previously demonstrated. The possibility of using haemoglobin oxidation in whole blood as an alternative assessment of glutathione peroxidase activity and hence of selenium status is proposed.

Introduction

A biological role for selenium was proposed by Rotruck *et al.* (1972, 1973) who showed that the element was involved in the glutathione (GSH)-dependent metabolism of hydro-peroxides. That selenium was in fact an integral component of the enzyme glutathione peroxidase (glutathione: hydrogen-peroxide oxidoreductase, E.C.1.11.1.9 —abbreviation GSH-Px) was confirmed by Flohe *et al.* (1973). Relationships between GSH-Px activity and blood selenium levels have since been demonstrated in sheep and cattle (Allen *et al.* 1976; Thompson *et al.* 1976; Wilson and Judson 1976), as well as in other species, and the measurement of this enzyme is now considered to be a good indication of selenium status.

The biochemical role of GSH-Px is in catalysing the breakdown of peroxides with a specific requirement for (reduced) GSH as a substrate (Mills 1957). The GSH-Px enzyme system has been shown by Mills (1957, 1959) and Rotruck *et al.* (1972) to function in the prevention of H_2O_2-induced oxidative damage to haemoglobin.

Many methods for the assay of GSH-Px are based on the coupled enzyme technique of Paglia and Valentine (1967). Board and Peter (1976) reported a spot test procedure that can be carried out with simple laboratory equipment.

In the experiments described here, the oxidation of haemoglobin in whole blood samples from sheep was induced by H_2O_2, while supplying GSH to allow the GSH-Px protective mechanism to operate. The quantitative relationship between the GSH-Px activity and the extent of oxidation was investigated.

Methods

Whole blood samples were collected from grazing wethers in evacuated tubes containing heparin. Shortly after collection, the packed cell volume of each sample was determined. The samples were then stored at $-20°C$ prior to assay, except for some of the preliminary experiments in which freshly collected samples were used.

Preliminary experiments were carried out to determine the optimum concentrations of H_2O_2 and GSH to be used in the procedure and to confirm the specificity of the reaction being monitored. In the final experiment, the extent of oxidation was measured in samples with a large range of GSH-Px activities.

GSH-Px activity

The GSH-Px activities of samples used in the preliminary experiments were estimated by the method of Board and Peter (1976) with some modifications as recommended by the authors (D. W. Peter, personal communication, 1977). For the purposes of these experiments, samples were selected that were considered to be of low, medium or high GSH-Px activity. The activities of these samples were approximately equivalent to 15, 50 and 200 enzyme units respectively, as determined below. In the final experiment, GSH-Px activity was determined by a technique based on that of Paglia and Valentine (1967). In this method, the reaction was carried out at 25°C, using 0·2 mM H_2O_2 as substrate and the calculated enzyme unit (e.u.) is equivalent to 1 μmol NADPH oxidized per minute per gram haemoglobin.

Haemoglobin Oxidation

The oxidation of haemoglobin was performed in phosphate buffer, 100 mM, pH 7·2, made up according to Beutler (1975) with the inclusion of 4 mM EDTA (disodium salt) and 0·1% (w/v) Saponin to promote rapid haemolysis. Solutions of NaN_3, GSH and H_2O_2 were made using the above buffer solution as diluent. The presence of EDTA ensures a low level of autoxidation of GSH, and catalase activity is inhibited by the inclusion of azide (Mills 1957). All reagents were analytical grade, and GSH was purchased from Calbiochem, Sydney.

A volume of 50 μl of the blood sample to be assayed was added to 2·35 ml of buffer, mixed and allowed to haemolyse. The sample haemolysate, azide, GSH and H_2O_2 solutions were added sequentially to form the reaction solution in an autoanalyser system (Technicon A.A.1). Mixing coils were employed to ensure adequate mixing of sample and reagents. The H_2O_2 was added last as this reagent initiates the oxidation reaction.

After the addition of the H_2O_2, reaction mixtures were passed through a delay coil in a water bath at 37°C for an incubation period of 13 min. Immediately after the incubation period, reaction solutions were passed through a colorimeter with a 625 nm filter for determination of optical density (O.D.). O.D. values were plotted with a chart recorder and read to the nearest 0·0025 unit.

All samples assayed underwent a blank reaction in which the H_2O_2 was replaced by buffer. The O.D. value of the blank reaction was subtracted to give the increase in O.D. for each sample assayed. This value was used as a measure of the extent of haemoglobin oxidation (Mills 1957; Rotruck *et al.* 1973).

The flow rates were 1·2 ml/min for sample haemolysates and 0·8 ml/min for additional reagents, using a sampling rate of 30 per hour and a sample to wash ratio of 2:1, giving a total assay volume of 4·8 ml. The reaction concentrations were 1·0 mM for azide and approximately 1 mg/ml for haemoglobin (depending on the haemoglobin content of the sample). Concentrations of H_2O_2 and GSH were varied as stated.

Results and Discussion

Peroxide and Glutathione Concentrations

The effect of varying the reaction concentration of H_2O_2 on the degree of oxidation in the samples is shown in Fig. 1*a*. In this case, the concentration of GSH was fixed at 6·6 mM. Fig. 1*b* shows the effect of varying GSH concentration, with approximately 0·1 mM H_2O_2.

From these results it was concluded that concentrations of approximately 0·1 mM H_2O_2 and 6·6 mM GSH appeared to give the best results. These gave submaximal oxidation at the lowest GSH-Px activity and an adequately measurable level at the highest activity.

Reaction Specificity

The breakdown of H_2O_2 by the GSH-Px system is specific in its requirement for GSH as a substrate (Mills 1957; Mills and Randall 1958; Cohen and Hochstein 1963).

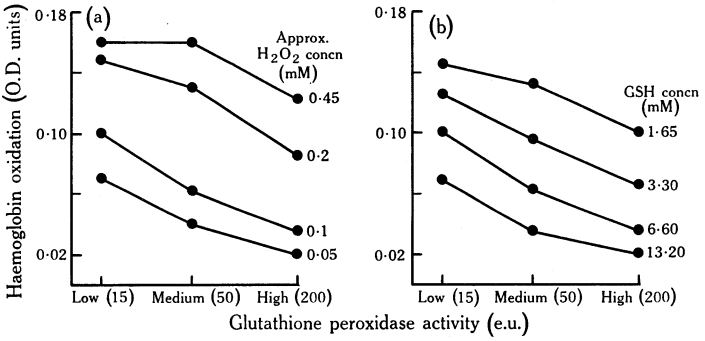

Fig. 1. Extent of haemoglobin oxidation, as measured by increase in optical density (O.D.) at 625 nm, in blood samples of low, medium or high glutathione peroxidase activity—approximate activities (on log scale) shown in parentheses in enzyme units (e.u.) equivalent to μmol NADPH oxidized per minute per gram haemoglobin. (*a*) Effect of varying H_2O_2 (approx. reaction concentrations as shown); (*b*) effect of varying GSH (reaction concentrations as shown).

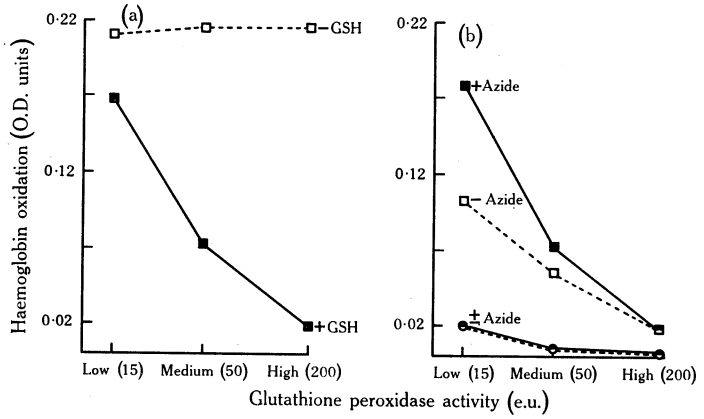

Fig. 2. Extent of haemoglobin oxidation, as measured by increase in optical density (O.D.) at 625 nm, in blood samples of low, medium or high glutathione peroxidase activity—approximate activities (on log scale) shown in parentheses in enzyme units (e.u.) equivalent to μmol NADPH oxidized per minute per gram haemoglobin. (*a*) Effect of GSH exclusion: ■ +GSH, □ −GSH; (*b*) effect of azide exclusion at two (approximate) concentrations of H_2O_2: ■ 0·1 mM H_2O_2 + azide; □ 0·1 mM H_2O_2 − azide; ● 0·01 mM H_2O_2 + azide; ○ 0·01 mM H_2O_2 − azide.

Fig. 2*a* shows the effect of exclusion of GSH from the reaction. These results and those shown in Fig. 1*b* confirm that the reaction being monitored is clearly dependent on GSH and that the degree of oxidation, with added GSH, is inversely related to the GSH-Px activity of the sample.

The inclusion of azide in the reaction is to inhibit catalase since this enzyme will also protect against haemoglobin oxidation (Mills 1957). The effect of exclusion of azide was examined at the optimal H_2O_2 concentration (approx. 0·1 mM), and also at a very low concentration (approx. 0·01 mM). These results, shown in Fig. 2*b*, are in agreement with the findings of Cohen and Hochstein (1963) that under physiological conditions of very low H_2O_2 concentrations, catalase does not play an important role in H_2O_2 breakdown relative to the GSH-Px system. Inhibition of catalase in the presence of azide was not detectable at the lower H_2O_2 concentration, but was considerable at the higher H_2O_2 concentration. Thus, azide is required in this assay system to ensure specificity.

The extent of oxidation seen in Figs 1*a* and 1*b* was less than that in Figs 2*a* and 2*b*. In the former case, previously frozen blood was used in the assay, whereas fresh blood was used in the latter. This is an apparent storage effect which requires consideration.

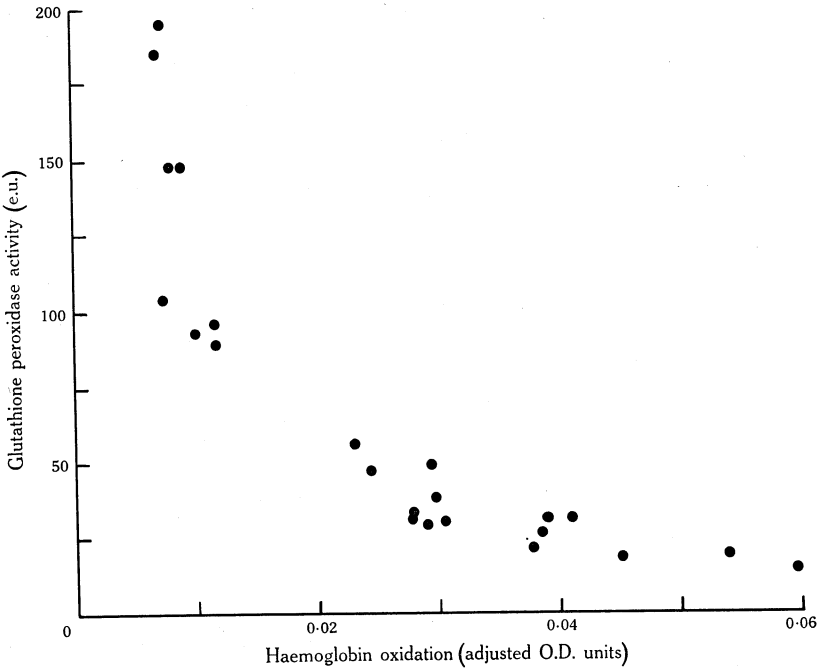

Fig. 3. Relationship between haemoglobin oxidation and glutathione peroxidase activity in whole blood samples. Haemoglobin oxidation is expressed as increase in optical density (O.D.) at 625 nm, adjusted for packed cell volume, using reaction concentrations of approximately 0·1 mM H_2O_2 and 6·6 mM GSH. GSH-Px activity is expressed as enzyme units (e.u.) equivalent to μmol NADPH oxidized per minute per gram haemoglobin.

Relationship between Haemoglobin Oxidation and GSH-Px Activity

Forty-two whole blood samples that had been stored frozen for 8 weeks after collection were assayed for haemoglobin oxidation, using reaction concentrations of approximately 0·1 mM H_2O_2 and 6·6 mM GSH, as determined by the preliminary experiments. At the same time, 23 of these samples were assayed for GSH-Px activity by the technique of Paglia and Valentine (1967). The relationship between the GSH-Px activity and extent of haemoglobin oxidation for these samples is shown in

Fig. 3. An adjustment was made to account for variation in haemoglobin content of different samples by multiplying the increase in O.D. by the packed cell volume.

The relationship shown in Fig. 3 was found to be well described using a logarithmic transformation on the activity values. The linear correlation between log (activity) and adjusted O.D. was highly significant ($r = -0{\cdot}942$, $P < 0{\cdot}01$). This correlation was not significantly different if the adjustment for packed cell volume was not made. However, there was little variation in the packed cell volumes of these samples.

The remaining 19 samples were assayed for GSH-Px activity after a further 3 weeks storage at 4°C but were not re-assayed at this stage for haemoglobin oxidation. The correlation between log (activity) at the second assay date and extent of oxidation measured previously was again highly significant ($r = -0{\cdot}961$, $P < 0{\cdot}01$). However, the slope of the regression function on this occasion was significantly different from the first. This result was due to an apparent loss in GSH-Px activity between the two assay dates as several samples were assayed on both occasions and indicated a decrease in activity of 10–20%. This loss in activity is thought to be due to a decrease in stability of the enzyme in the haemolysed state after freezing.

Conclusion

These results have demonstrated the quantitative relationship between GSH-Px activity and H_2O_2-induced haemoglobin oxidation in whole blood samples. The high degree of correlation observed shows that the oxidation assay procedure may be a practical technique for assessing GSH-Px activity in whole blood if elaborate spectrophotometric facilities are not available. The technique may also be of use for other studies involving haemoglobin oxidation.

Acknowledgments

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