OXIDATION IN THE NADP SYSTEM AND RELEASE OF GSSG FROM HEMOGLOBIN-FREE PERFUSED RAT LIVER DURING PEROXIDATIC OXIDATION OF GLUTATHIONE BY HYDROPEROXIDES

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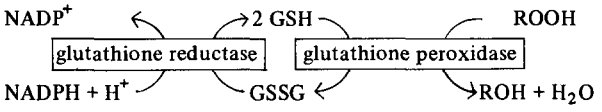
1. Introduction

Glutathione represents the major cellular pool of mobile sulfhydryl groups. The redox couple, 2GSH/GSSG, is linked to the NADP system by glutathione reductase (EC 1.6.4.2), and to the hydroperoxide systems by glutathione peroxidase (EC 1.11.1.9):



scheme 1

Both of these enzyme activities have been studied extensively in recent years in rat liver homogenates and subcellular fractions [1–6]. In addition to H_2O_2, several organic hydroperoxides were reported as active substrates for rat liver glutathione peroxidase [2]; some of these, e.g. t-butyl hydroperoxide or cumene hydroperoxide, do not react with catalase, and the corresponding alcohols do not react with alcohol dehydrogenase. Therefore, a selective transition in the 2GSH/GSSG redox system is effected in intact liver cells by these substrates.

Abbrevations: GSH = reduced glutathione; GSSG = oxidized glutathione

On this basis, the investigation into GSH-dependent hydroperoxide metabolism is used to obtain further insight into the state of the NADP system and its linkage to connected metabolic pathways. This is achieved by monitoring the redox state of cellular NADPH by surface fluorescence, and by analysis of the perfusate for peroxide, GSSG, lactate, pyruvate, glucose, pH and oxygen, using an open system of hemoglobin-free perfused rat liver [7, 8].

2. Materials and methods

2.1. *Materials*

Glutathione peroxidase was prepared according to [9]. All other enzymes as well as all coenzymes, GSSG and Na-pyruvate were from Boehringer, Mannheim. L-lactic acid was from Roth, Karlsruhe. 5,5′-Dithiobis (2-nitrobenzoic acid) was from Serva, Heidelberg. t-Butyl hydroperoxide and cumene hydroperoxide were from Elektrochemische Werke München, Höllriegelskreuth. Ethyl hydroperoxide was from Ferrosan, Malmö. All other chemicals were from Merck, Darmstadt.

2.2. *Liver perfusion*

Hemoglobin-free perfusion of livers from male Wistars rats, 140–180 g weight, fed on stock diet,

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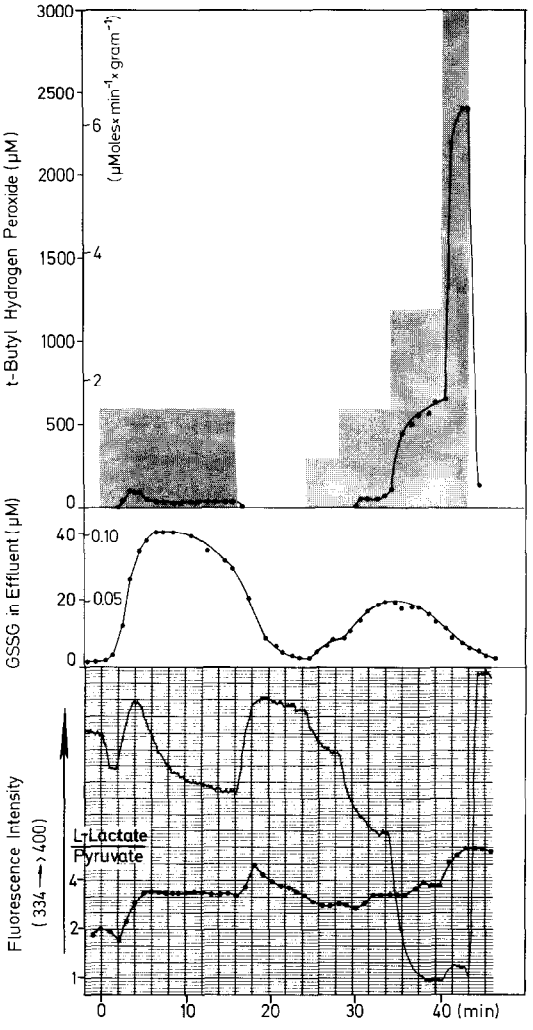

Fig. 1A. Response of hemoglobin-free perfused liver to infusion of t-butyl hydroperoxide. Entering concentrations are shown as shaded columns, marking the respective time intervals. Peroxide concentration in effluent is shown by solid circles. GSSG in effluent perfusate is shown in center panel. Concentrations are converted to rates (μmoles per g per min, inner scale) from perfusate flow (20 ml per min in this experiment) and liver wet weight. Pyridine nucleotide-specific surface fluorescence and [lactate]/[pyruvate] in effluent are shown in bottom panel. [lactate]/[pyruvate] in entering perfusate was held constant at 1.

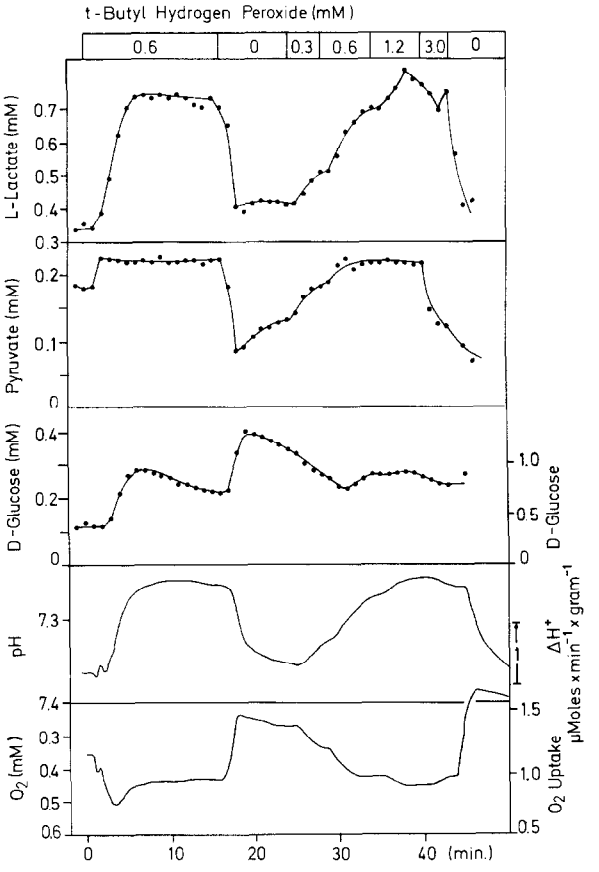

Fig. 1B. Effluent perfusate analyses for lactate, pyruvate, glucose, pH and O_2. Same experiment as fig. 1A. Rates for glucose and H^+ output and for overall O_2 uptake are given by the right hand scales.

was performed with bicarbonate-buffered solution consisting of 115 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM Na_2SO_4, 2.5 mM $CaCl_2$, 0.2 mM Na-L-lactate, 0.2 mM Na-pyruvate, 25 mM $NaHCO_3$, equilibrated with an oxygen–carbon dioxide mixture (95/5, v/v) in a temperature-regulated (33° on liver surface) rotating cylinder oxygenator as described [7, 8]. The perfusate passed through the liver via vena portae and vena cava at a rate of 3–3.5 ml per min per g of liver; it was not recirculated (open system). Additions were made by infusion of stock solutions using precision micropumps.

2.3. *Perfusate analyses*

Perfusate samples were collected at 30 sec intervals and assayed for GSSG [10], hydroperoxides, l-lactate, pyruvate, glucose and lactate dehydrogenase using appropriate simple or coupled enzymatic assays. [O_2]

and pH in perfusate were monitored continuously as described [7, 8].

2.4. *Fluorometry*

Pyridine nucleotide-specific fluorescence (excitation at 334 nm, emission >400 nm) was continuously monitored from the surface of a liver lobe as described [7].

3. Results

3.1. *t-Butyl hydroperoxide utilization*

In the experiment of fig. 1, the entering perfusate was initially 0.6 mM with respect to t-butyl hydroperoxide (top panel in fig. 1A, outer scale, shaded column from 0 to 16 min), corresponding to 1.55 μmoles peroxide reaching the liver per g per min (inner scale). It is seen that only about 3% of the peroxide appears in the effluent perfusate (solid circles). As shown in the time interval from 24 to 43 min, lower peroxide concentrations (0.3 mM) result in no appearance of peroxide in the effluent perfusate, while higher concentrations (1.2 mM and 3 mM) do not lead to an increased rate of removal of peroxide from the perfusate. Thus, under these conditions rat liver is capable of removing t-butyl hydroperoxide from the perfusate at maximal rates of 1.5 μmoles of peroxide per g liver wet weight per min.

3.2. *Release of GSSG*

In presence of t-butyl hydroperoxide, oxidized glutathione is released from the liver (center panel in fig. 1A). The maximal rate of release of GSSG from the liver is 0.1 μmole GSSG per g liver wet weight per min. This corresponds to about 4% of total GSH + GSSG, which was found to be 5.28 ± 0.65 μmoles GSH equivalents per g liver wet weight (n = 4) with the method described in [10]. In the experiment of fig. 1, 3/4 of total liver GSH + GSSG were found in the total perfusate.

The appearance of GSSG is clearly distinguished from possible general cell disintegration by the peroxide by the reversibility of the effect (fig. 1A), and by the profile of appearance of a marker enzyme for cytosol, lactate dehydrogenase (not shown); until 30 min, activity of this enzyme was < 1 mU per ml of perfusate, and then gradually increased to 50 mU per ml towards the end of perfusion.

3.3. *Pyridine nucleotide-specific surface fluorescence*

The ratio, [lactate] / [pyruvate], was held constant at 1 in the entering perfusate (see Methods) for stabilization of the cytosolic NAD redox potential (cf. [7]). In the bottom panel of fig. 1A, both the surface fluorescence and the [lactate] / [pyruvate] in effluent perfusate (solid circles) are shown. Considering first the time interval from 24 to 43 min, it is seen that fluorescence intensity decreases markedly during t-butyl hydroperoxide metabolism and that this decrease is dependent upon the concentration of hydroperoxide. This effect is attributable to an oxidation in the NADP system as is expected from scheme 1.

However, consistently a more complex transition is observed during the initial peroxide infusion, as shown in the time interval from 0 to 16 min. The transient rise of fluorescence intensity coincides with the transition of [lactate] / [pyruvate] from 2 to an increased plateau level of 3.4. Therefore, in this period a transition of the cytosolic NAD system to a more reduced steady state superposes with the oxidation in the NADP system.

3.4. *General metabolic response*

In addition to the lactate and pyruvate profiles, fig. 1B also gives the glucose output. Interestingly, an increased glucose output is observed simultaneously with the increased lactate output, in agreement with an increased rate of glycogenolysis.

The metabolic response to the peroxide is further evidenced by changes of pH in effluent perfusate and of oxygen concentration in effluent perfusate (fig. 1B). The extra production of acid equivalents is nearly fully accounted for by the release of GSSG, lactate and pyruvate. There is a reversible decrease of overall uptake of oxygen by the liver in presence of peroxide. It may be mentioned that infusion of t-butanol (2 mM) did not lead to changes of fluorescence intensity, effluent pH or oxygen uptake.

3.5. *Other hydroperoxides*

Cumene hydroperoxide effects are similar to those obtained with t-butyl hydroperoxide. The fluorescence response and the appearance of GSSG in presence of 0.3 mM cumene hydroperoxide are demonstrated in fig. 2. Ethyl hydroperoxide also leads to release of GSSG from the liver (not shown), but in other parameters, e.g. surface fluorescence, there are differences due to the effects of the product, ethanol.

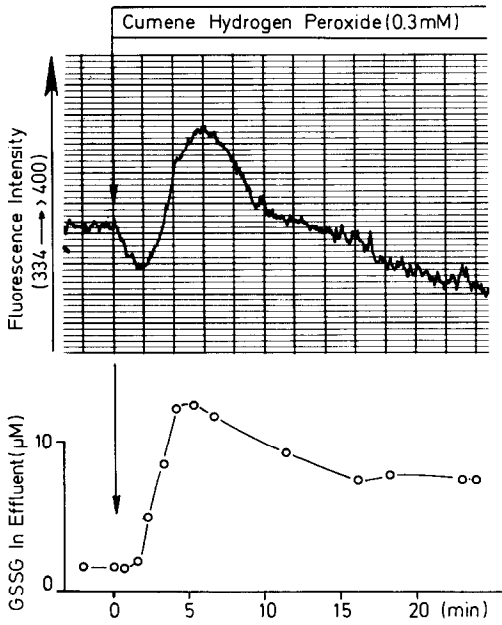

Fig. 2. Responses of surface fluorescence and of GSSG release during infusion of cumene hydroperoxide into the entering perfusate of hemoglobin-free perfused rat liver.

Infusion of hydrogen peroxide also leads to a decrease of fluorescence intensity and to a release of GSSG from the liver (fig. 3). The initial release of extra lactate and glucose is also observed with H_2O_2. The substantial increase of $[O_2]$ in effluent perfusate, particularly the higher increment in the step from 0.5 to 1 mM H_2O_2, indicates the production of oxygen by catalatic action of catalase under these conditions.

4. Discussion

Hemoglobin-free perfused rat liver is a suitable experimental system for investigation of the interrelations between glutathione-dependent peroxide metabolism on the one hand and the NADP system with the linked metabolic network on the other hand, in the intact hepatocyte. t-Butyl hydroperoxide proved a suitable model substance, it being reduced specifically by glutathione peroxidase (from erythrocytes) at a rate of about 7.5×10^6 M^{-1} sec^{-1} [11].

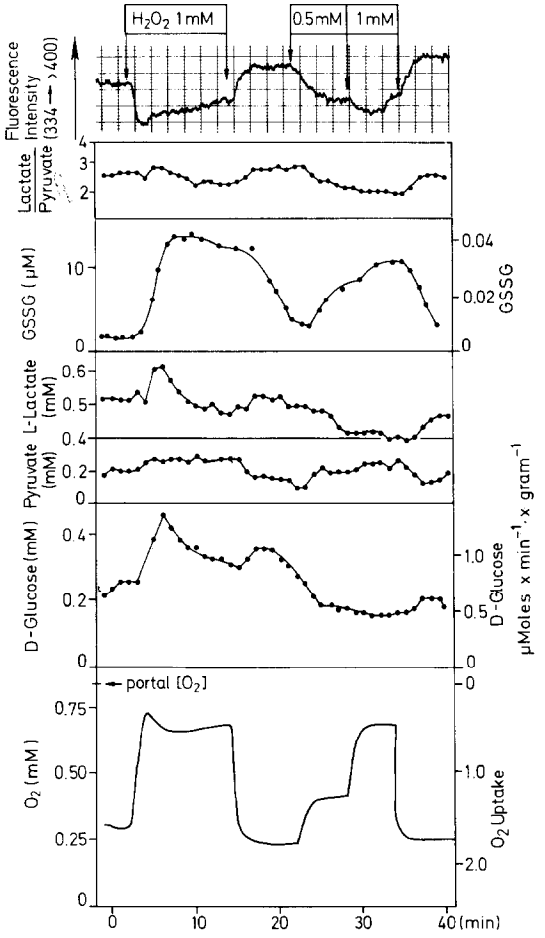

Fig. 3. Response of hemoglobin-free perfused rat liver to infusion of hydrogen peroxide. Concentrations are given in the left hand scales, corresponding rates are given in the right hand scales.

Like in erythrocytes [12] or lens [13], GSSG is released from the cells during increased rates of peroxide reduction at the glutathione peroxidase. Obviously, GSH regeneration at glutathione reductase is insufficient to keep GSSG at the calculated equilibrium concentration of about 10^{-8} M, with an assumed GSH concentration of 6 mM [14]. However, the activity level of both of these enzymes may exceed the observed maximal rate of peroxide reduction, 1.5 μmoles g per min. Glutathione peroxidase, at optimal GSH concentrations, is present at about 50-fold excess as calculated from measurements on rat liver homogenate [3].

Glutathione reductase activity is 10 μmoles of GSSG reduced per g of liver per min at 30° [4], assayed at 0.3 mM NADPH and 2.5 mM GSSG. The Michaelis constants for the enzyme were reported to be 3 μM NADPH and 50 μM GSSG [6]. Thus, the possibility exists that glutathione reductase does not operate under saturation conditions *in situ*. More likely, the rate limitation may be exerted at the level of NADPH regeneration. The low intensity of fluorescence at 38 min in fig. 1A corresponds to 50% of the initial intensity, which is much lower than the NADP-linked decrease of fluorescence observed during high rates of mixed function oxidation [15]. Preliminary data on total tissue levels indicate a substantial decrease of NADPH and an increase of NADP in presence of the peroxide. It will be of interest to observe the indicator metabolite couple, malate/pyruvate, as it was in [15], although the activity level of 1.3 U per gram at 38° for malic enzyme [14] points toward non-equilibrium under these conditions.

The transient rise of fluorescence intensity, attributed to the more reduced steady state in the cytosolic NAD system, may find its explanation by the increased rate of glycogenolysis. With the energy requirement for increased glycogenolysis, the decreased phosphorylation potential would equilibrate with the NAD system at glyceraldehyde phosphate dehydrogenase and phosphoglycerate kinase [16].

The release of GSSG from the liver in presence of exogenous H_2O_2 (fig. 3) substantiates the concept that, in addition to catalase, glutathione peroxidase plays a role in H_2O_2 metabolism in the hepatocyte. Such conclusions were also drawn, for example, from experiments with rat liver homogenates [5]. In view of the peroxisomal localisation of catalase [17], it appears appropriate to expect that H_2O_2 generated in extraperoxisomal compartments may have two alternative metabolic pathways functional during normal conditions of NADPH supply.

Acknowledgements

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