Biochemical Pharmacology Vol 35 No 13 pp 2268–2270 1986
Printed in Great Britain

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd

Importance of the glutathione redox cycle for the resistance of lung epithelial cells against a polymorphonuclear leukocyte-mediated oxidant attack

(*Received* 7 *October* 1985, *accepted* 16 *January* 1986)

The interaction of stimulated polymorphonuclear leukocytes (PMN) with lung cells is considered to play a major role in the pathogenesis of lung disease. PMN-mediated cell damage has been attributed to reactive oxygen metabolites as the major cytotoxic species [1, 2]. In previous studies we have shown that lung epithelial cells are injured by stimulated PMN through a H_2O_2 dependent mechanism [3]. Target cells will defend themselves by destroying the attacking hydrogen peroxide. Two major enzyme systems are responsible for H_2O_2 catabolism in mammalian cells [4]: glutathione peroxidase, a selenoenzyme which utilizes reduced glutathione to metabolize hydrogen peroxide and catalase, a hemoprotein which converts hydrogen peroxide to H_2O and O_2.

In the present study cytotoxic experiments in lung epithelial cells were carried out designed to determine the relative importance of catalase and glutathione peroxidase as intracellular defense mechanisms against lysis by extracellularly generated hydrogen peroxide.

Materials and methods

Lung epithelial cells derived from a clone of rat type II pneumocytes were maintained in tissue culture as previously described [3]. Confluent monolayers were processed for determinations of cytotoxicity and of enzyme activities. For cytotoxicity studies lung epithelial cell monolayers were labeled with 3 μCi ^{51}Cr ($Na_2{}^{51}CrO_4$, New England Nuclear) overnight, washed and divided into four groups: one group was incubated for 10 min with 125 μg/ml 1,3-bis-(chloroethyl)-1-nitrosourea (BCNU, Bristol Lab, Syracuse, NY), a potent inhibitor of glutathione reductase [5]. The second group received for 90 min 50 mM 3-amino-1,2,4-triazole (Sigma Chem. Co., St. Louis, MO) a potent inhibitor of catalase [6]. The third group received both reagents and the fourth one served as control. 2.5–16 million PMN (obtained as previously described [3]), were added to the differently treated lung epithelial cells in Hanks' balanced salt solution (11 mM glucose). This range of PMN provided an effector/target cell ratio between 1:1 and 64:1. PMN were stimulated with 0.25 μg/ml phorbol myristate acetate. Following a 4 hr incubation PMN-mediated H_2O_2 related cytotoxicity was determined by quantitating ^{51}Cr release from lung epithelial cells as previously described [3], i.e. by a simple, quantitative, and well established approach to cytotoxicity [7]. For determination of enzyme activities confluent lung epithelial cell monolayers were dispersed, cells were disrupted by sonication, and an aliquot was used for determination of protein according to Lowry *et al.* [8], of the activity of glutathione peroxidase (EC 1.11.1.9) according to Paglia and Valentine [9], of glutathione reductase (EC 1.6.4.2) according to Carlberg and Mannervik [10], and of catalase (EC 1.11.1.6) according to Beers and Sizer [11].

Glutathione peroxidase deficient lung epithelial cells were obtained by depleting them of selenium. Usually the serum in the medium provides the selenium to cultured cells [12]. While serum free growth would be ideal for selenium depletion preliminary experiments established that 0.75% serum was the lowest concentration capable of consistently maintaining cell viability. Lung epithelial cells grown in 0.75% serum, in 0.75% serum supplemented with 27 nM Na_2SeO_3, and in 10% serum for up to 10 days were processed for enzyme studies or for cytotoxicity studies using glucose oxidase as the source of H_2O_2.

Data were analysed by the two tailed Students' *t* test for unpaired samples [13].

Results

Preincubation of lung epithelial cells with BCNU consistently inhibited glutathione reductase activity by more than 95% while catalase activities were unaffected by this pretreatment. Preincubation of the cells with aminotriazole resulted in a consistent inhibition of their catalase activity by 95–98% without any impairment of the glutathione peroxidase- or reductase activities. As demonstrated in Fig. 1 lung epithelial cells with almost no functional glutathione reductase activity displayed a dramatically increased susceptibility to a PMN-mediated oxidant attack. Virtually complete protection of these highly susceptible target cells was afforded when exogenous catalase was added to this cytotoxic system. Following inhibition of the (endogenous) catalase of lung epithelial cells there was an only minor increase in susceptibility of target cells to a PMN-mediated attack. Combined inhibition of glutathione reductase- and catalase activity resulted in the same degree of enhanced target cell damage as that seen following inhibition of glutathione reductase alone.

As a second way to interrupt the GSH-redox cycle glutathione peroxidase was inhibited by taking advantage of the selenium dependency of this enzyme. Culture of confluent lung epithelial cells in medium supplemented with 0.75% serum resulted in a time dependent decline in glutathione peroxidase activity reaching 6% of the original activity after 7–10 days (Fig. 2). Cells grown under the same low serum conditions supplemented, however, with 27 nM selenium displayed glutathione peroxidase activities not different from lung epithelial cells grown in medium plus 10% serum. Catalase activities were not different in either cell group. As demonstrated in Fig. 3 there was a substantially increased susceptibility of glutathione peroxidase deficient lung epithelial cells against an oxidant attack by extracellularly generated hydrogen peroxide.

Discussion

The demonstration of a toxic effect of PMN-derived or chemically generated hydrogen peroxide on lung epithelial cells confirms our previous observation [3] and is in agreement with similar results obtained in lung fibroblasts, endothelial cells, and different tumor cells [1, 2]. Removal of the toxic agent 60 min (but not 90 min) after start of the experiment rescues the lung epithelial cells from lethal injury. This apparently indicates a delicate balance between the attacking hydrogen peroxide and defending mechanisms of the target cells in terms of metabolism of the toxic agent or of repair of the toxic lesion before this balance finally tips over. This balance can dramatically be altered by an impairment of the H_2O_2 catabolizing capacity of the lung epithelial cells. Inhibition of the activity of the GSH-redox cycle by interference with the activity of glutathione reductase or of glutathione peroxidase renders the lung epithelial cells at least 30 times more susceptible to a PMN-mediated oxidant attack. Similar observations have been made in endothelial cells by Harlan *et al.* [14]. Interestingly catalase, an enzyme in lung epithelial cells with a much higher H_2O_2 metabolizing capacity than the GSH-redox cycle, provides only minimal protection against a flux of extracellularly generated H_2O_2. Possible explanations are (I) a H_2O_2 related generation of toxic lipid peroxides which exclusively can be metabolized by the GSH-redox cycle [4], (II) the higher K_m of catalase for H_2O_2 [15], or (III) a compartimentalisation within the cell with catalase and the GSH-redox cycle metabolizing hydrogen peroxide of different sources in the cell as has

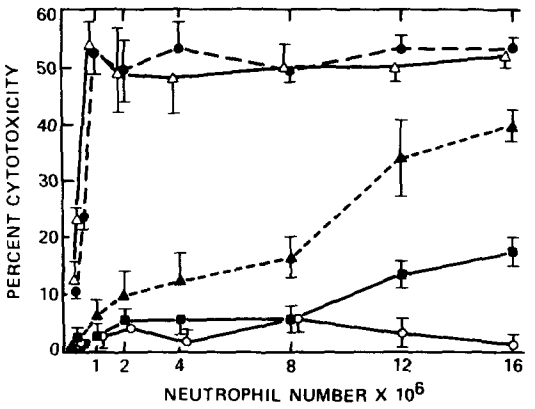

Fig 1 Substantially enhanced cytotoxicity of PMN-derived hydrogen peroxide on confluent lung epithelial cells following an impairment of the glutathione reductase but not of catalase Note that in the presence of exogenous catalase target cells are completely protected against a PMN-mediated attack Data presented are mean (±S E) of 5 experiments ■—■ target cells, no pretreatment, △—△ target cells, pretreated with BCNU, ▲——▲ target cells, pretreated with aminotriazle (AT), ●——● target cells, pretreated with BCNU and AT, ○—○ target cells, pretreated with BCNU, then addition of exogenous catalase.

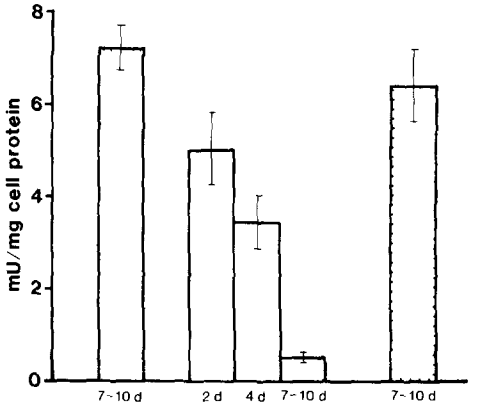

Fig 2 Time dependent decrease of glutathione peroxidase activity in lung epithelial cells cultured in medium containing only 0 75% serum (open bars) Cells grown in 0 75% serum supplemented with 27 nM selenium (right bar) showed no different glutathione peroxidase activities as compared to cells kept in the "regular" medium containing 10% serum (left bar) Data presented are mean (±S E) of 6 experiments

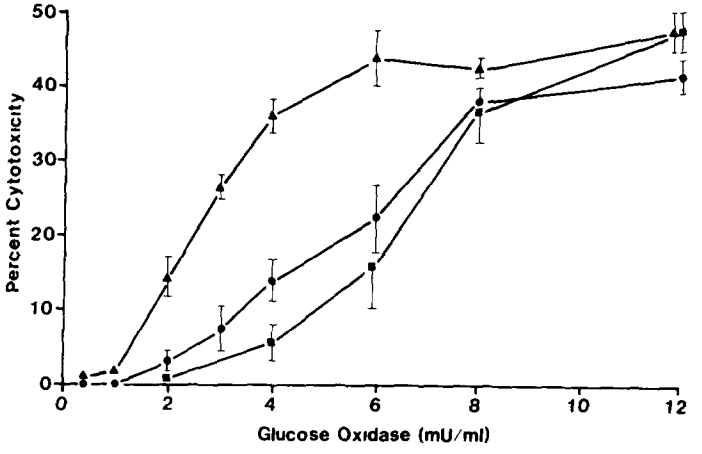

Fig 3 Enhanced hydrogen peroxide mediated cytotoxicity in glutathione peroxidase deficient lung epithelial cells Lung epithelial cells grown in 0 75% serum for 10 days (▲—▲) displayed glutathione peroxidase activity which were 6% of that measured in cells grown in 0 75 serum supplemented with 27 nM Na_2SeO_3 (●—●) or in medium containing 10% serum (■—■) A continuous flux of extracellularly generated hydrogen peroxide was provided by glucose oxidase Data presented are mean (±S E) of 6 experiments

been shown for hepatocytes [16] Additional studies are required to distinguish among these different possibilities

In summary, PMN induced oxidant damage of lung epithelial cells, known to be a H_2O_2 dependent mechanism, is greatly enhanced following an impairment of the hydrogen peroxide metabolism of the target cells The GSH-redox cycle is substantially more important than catalase in providing protection against this sort of oxidant attack

Department of Clinical Chemistry and Pathobiochemistry
University of Giessen
Federal Republic of Germany
NORBERT SUTTORP

Department of Medicine
School of Medicine and Veterans Administration Medical Center
Stanford University
Palo Alto
CA 94304, U S A
LAWRENCE M SIMON

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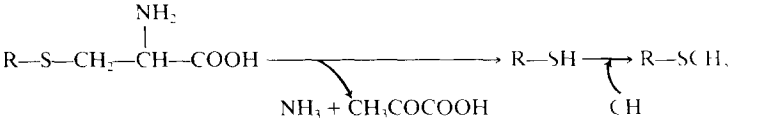
Biochemical Pharmacology Vol 35 No 13 pp 2270–2272 1986
Printed in Great Britain

0006-2952/86 $03.00 + 0.00
Pergamon Journals Ltd

A novel pathway for formation of thiol-containing metabolites from cysteine conjugates

(*Received* 25 *October* 1985, *accepted* 31 *January* 1986)

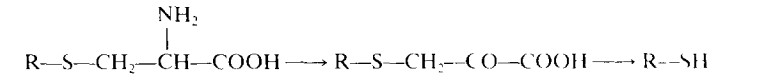
During the past decade the formation of methylthio-containing metabolites has been reported for numerous xenobiotics including drugs, herbicides, and other organic compounds (reviewed by Tateishi [1, 2] and Stillwell [3]) From the extensive studies in our laboratories [4–7] and others [8, 9], various types of methylthio-containing metabolites were shown to be generated from the corresponding cysteine conjugates via thiols (Scheme 1)



Scheme 1

Thus the C—S bond of cysteine conjugates is firstly cleaved by the action of C—S lyases (which are found in mammalian liver, kidney and some kinds of intestinal microorganisms) to give thiols together with equimolar amounts of pyruvic acid and ammonia [5, 7] The thiols thus formed are subsequently methylated by *S*-methyltransferases [5, 10]

In addition to this pathway of formation of thiols we have now found that thiols may be generated from cysteine conjugates via an alternative pathway (Scheme 2) in which the intermediate formation of thiopyruvic acid conjugates is most likely to be involved



Scheme 2

In the present communication we will describe the evidence for this novel pathway

Materials and methods

Materials S-(*p*-Bromophenyl)-L-cysteine and *p*-bromophenyl-3-thiopyruvic acid were synthesized by the method described in our previous report [7] *p*-Bromobenzenethiol was purchased from Aldrich (U S A) All other reagents used in the present study were of analytical special grade and commercially available

The liver was excised from male Sprague–Dawley rats (8 weeks old) and homogenized in 2 vol of ice-chilled 50 mM of potassium phosphate buffer (pH 7 4) The cytosol (105,000 *g* supernatant) and microsomal fractions were obtained by published methods [5] The following *in vitro* experiments were carried out with these enzyme preparations

Deamination of S-(p-*bromophenyl*)-L-*cysteine* The reaction mixture contained in a total volume of 0 5 ml S-(*p*-bromophenyl)-L-cysteine (0 5 μmole) 0 3 ml of potassium phosphate buffer (5 μmole pH 7 4) and 0 2 ml of the enzyme preparation (8 mg protein) The mixture was incubated at 37° for 30 min and the reaction terminated by an addition of 0 5 ml of acetonitrile at 0° After centrifugation a portion of the supernatant was applied to an h p l c column (YMC A-312 ODS 6 × 150 mm Yamamura Chemical, Osaka, Japan) and the column eluted with a solvent system of methanol/water/acetic acid (5 4 1 by vol) at a flow rate of 1 5 ml/min *p*-Bromophenyl-3-thiopyruvic acid which emerged as a single sharp peak at 5 6 min under these conditions was quantitatively analyzed by monitoring the uv absorbance at 254 nm

C—S cleavage of p-*bromophenyl-3-thiopyruvic acid* The reaction mixture contained in a final volume of 0 2 ml *p*-bromophenyl-3-thiopyruvic acid (0 2 μmole) as a substrate dithiothreitol (1 μmole) potassium phosphate buffer (10 μmole pH 7 4) and 0 04 ml of the enzyme preparation The reaction mixture was incubated at 37° for 1 hr under anaerobic conditions The incubation was terminated by an addition of 0 2 ml of acetonitrile containing *p*-fluoroben-