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# S-D-LACTOYLGLUTATHIONE IN RESTING AND ACTIVATED HUMAN NEUTROPHILS

P.J. Thornalley*, V. Della Bianca, P. Bellavite, and F. Rossi

*Pharmaceutical Research Institute, Aston University, Birmingham B4 7ET, U.K.
and Istituto di Patologia Generale, Universita di Verona,
Strada le Grazie, 37134 Verona, Italy

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Zymosan particles opsonised with human serum factors functionally activate human neutrophils and induce a substantial modification of the human neutrophil cytoslic glyoxalase system. The activity of glyoxalase I increases and the activity of glyoxalase II decreases by 20-40% of their resting cell activities during the initial 10 min of activation. The cellular concentration of the glyoxalase intermediate S-D-lactoylglutathione increases by ca. 100% of resting cell levels during this period. This modification may be related to the ability of S-D-lactoylglutathione to stimulate the assembly of microtubules. © 1987 Academic Press, Inc.

The glyoxalase system catalyses the conversion of methylglyoxal to D-lactic acid via the putative intermediate S-D-lactoylglutathione (1). It comprises two enzymes and a catalytic amount of reduced glutathione. Glyoxalase I catalyses the formation of S-D-lactoylglutathione from the non-enzymatically formed hemimercaptal adduct of methylglyoxal and reduced glutathione (2). $CH_3COCHO + GSH \rightleftharpoons CH_3COCH(OH)SG \longrightarrow CH_3CH(OH)CO\text{-}SG$. Glyoxalase II (EC 3.1.2.6) catalyses the hydrolysis of S-D-lactoylglutathione to D-lactic acid and reduced glutathione (3), $CH_3CH(OH)CO\text{-}SG + H_2O \rightarrow CH_3CH(OH)CO_2H + GSH$. It is the only known route for the metabolism of S-D-lactoylglutathione and occurs widespread through plant and animal tissue (4).

The cellular concentration of S-D-lactoylglutathione has hitherto not been reported. This little-studied glutathione metabolite has been shown to stimulate the polymerisation of tubulin *in vitro*. (5) and to potentiate the release of histamine from human leukocytes (6). It may also be involved in the induction of microtubule assembly during the functional activation and lysosomal degranulation of human neutrophils by the tumour-promoting phorbol diester, 12-O-tetradecanoylphorbol-13-acetate (TPA) (7,8) and zymosan opsonised with human serum (9).

To test this hypothesis, we have examined the activities of glyoxalase I and glyoxalase II and the concomitant concentrations of reduced glutathione

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and S-D-lactoylglutathione (glyoxalase II substrates) during the functional activation of human neutrophils by opsonised zymosan. We report here results from a study of the neutrophil response from four different donors.

## MATERIALS AND METHODS

Human neutrophils (>95%) were prepared by dextran sedimentation and centrifugation on Ficoll-Hypaque gradients (10) using citrate anticoagulated venous blood obtained from healthy adult donors. After hypo-osmotic lysis from contaminating erythrocytes, the cells were suspended in medium containing 110 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 25 mM HEPES, pH 7.4 with 5 mM glucose. Neutrophils ($10^7$/ml) were incubated with 3 mg/ml opsonised zymosan (11) and without stimulant. Samples were withdrawn after the time intervals indicated and injected into 10 volumes of ice-cold incubation buffer. Cells were sedimented by centrifugation, re-suspended in 10 mM HEPES, pH 7.0 with 0.2% Triton X100 and ruptured by sonication (100 W, 20s). The membranes were sedimented by centrifugation (50,000 g, 30 min) and the supernatant was assayed for glyoxalase activity.

Assay of Glyoxalase I Activity - This was determined by measuring the initial rate of formation of S-D-lactoylglutathione from hemimercaptal, followed spectrophotometrically at 240 nm, $\Delta\epsilon = 2.86\ mM^{-1}cm^{-1}$ (12). Hemimercaptal (1.33 mM) was prepared by incubating 2 mM methylglyoxal with 2 mM GSH in 100 mM sodium phosphate, pH 6.6 and 37 C, for 30 min. The rate for blanks (sonication buffer) was subtracted from all assays and the percentage change in glyoxalase I activity in activated cells relative to resting cells was calculated.

Assay of Glyoxalase II Activity - This was determined by measuring the initial rate of decrease in the concentration of S-D-lactoylglutathione followed spectrophotometrically at 240 nm, $\Delta\epsilon = 3.1\ mM^{-1}cm^{-1}$ (12) in 50 mM Tris/HCl, pH 7.4 and 37 C. S-D-Lactoylglutathione was prepared and purified by the method of Uotila (13). The rate of blanks (sonication buffer) was subtracted from all assays and the percentage change in glyoxalase II activity relative to resting cells was calculated.

Assay of reduced glutathione and S-D-lactoylglutathione - Neutrophils ($10^7$) were sedimented in cold buffer by centrifugation. The supernatant was immediately removed and the pellet was extracted with 0.25 ml 10% trichloroacetic acid in 0.02% HCl plus 0.25 ml 0.25% Triton X100. After holding on ice for 20 min., the samples were centrifuged to sediment the precipitate. The supernatent was removed and the precipitate was extracted once more with a further aliquot of trichloroacetic acid. The combined extracts were extracted with 5 x 1 volume water-saturated diethylether; residual ether was dispersed with nitrogen gas. The final extract was assayed for reduced glutathione by the method of Davies et al. (14), which involves the spectrophotometric assay of the chromophore S-(2,4-dinitrophenyl)-glutathione generated from 1-chloro-2,4-dinitrobenzene with glutathione-S-transferase. S-D-Lactoylglutathione was determined sequentially to reduced glutathione by measuring the further release of reduced glutathione from the cell extract upon the addition of glyoxalase II. Both enzymatic assay steps were incubated for one hour at 37$^{o}$C prior to spectrophotometric measurement. Assays were blanked and calibrated by assaying sonication buffer and solutions of known reduced glutathione and S-D-lactoylglutathione concentration respectively. Recovery was consistently over 95% for reduced glutathione and S-D-lactoylglutathione.

## RESULTS

### The Concentration of S-D-Lactoylglutathione in Resting Neutrophils

S-D-Lactoylglutathione was assayed in neutrophils prepared from blood of five different healthy donors. The concentration of S-D-lactoylglutathione in resting human neutrophils was found to be in the range 0.2-0.4 nmol/$10^6$ cells see Table 1. The mean concentration of S-D-lactoylglutathione in the human neutrophil was 0.32 nmol/$10^6$ cells. The mean concentration of reduced glutathione was 0.97 nmol/$10^6$ cells. Previous estimates of the concentration of reduced glutathione in human neutrophil have been in the range 0.7-1.3 nmol/$10^6$ cells (15,16,17). Our estimate corroborates within this range of reported estimates. Our estimation of the concentration of S-D-lactoylglutathione indicates that it is ca. 30% of the concentration of reduced glutathione (cf. oxidised glutathione which is ca. 2% reduced glutathione concentration in resting cells (16)).

### Modification of Glyoxalase Activities and S-D-Lactoylglutathione Status During Treatment with Opsonised Zymosan

When neutrophils are challenged with serum-treated zymosan particles, the cells are activated to chemotaxis, phagocytosis, secretion of specific and azophilic granule content, aggregation and production of oxygen free radicals through a superoxide-forming NADPH oxidase (17-24). During this activation of neutrophils, there is a substantial change in the neutrophilic glyoxalase system: the activity of glyoxalase I increases and the activity of glyoxalase II decreases (Figure 1). There is a concomitant decrease in the concentration of reduced glutathione and an increase in the concentration of S-D-lactoylglutathione (Figure 2). These changes are dependent on the presence of the zymosan stimulant and the duration of the activation period. The activity of glyoxalase I increased and the activity of glyoxalase II decreased by ca. 20-40% of their resting cell activities over the initial 10 min. activation period.

Table 1. S-D-Lactoylglutathione and Reduced Glutathione in Resting and Activated Human Neutrophils

| | Concentration (nmol/$10^6$ cells) | |
|---|---|---|
| | RESTING | ACTIVATED[a] |
| Metabolite | $\bar{x}$ ± s.e.m.(n) | $\bar{x}$ ± s.e.m.(n) |
| Reduced Glutathione | 0.97 ± 0.03 (4) | 0.51 ± 0.11 (4) |
| S-D-Lactoylglutathione | 0.32 ± 0.05 (4) | 0.68 ± 0.05 (4) |

a Concentrations determined after 10 min incubation with 3 mg/ml opsonised zymosan, as described in Materials and Methods section.

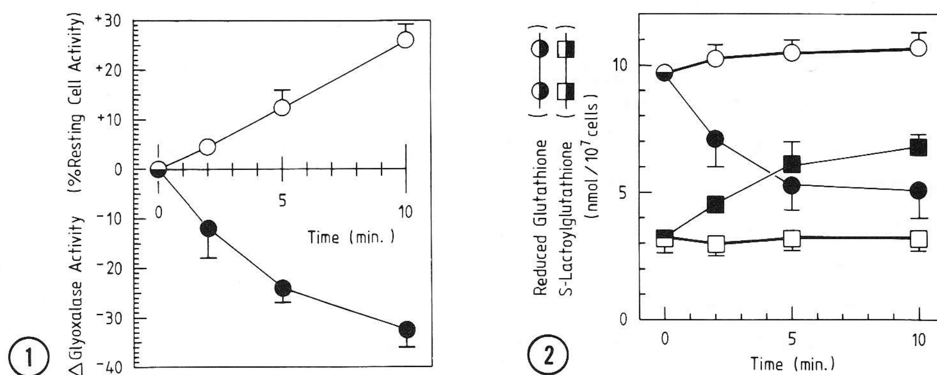


Figure 1. Changes in glyoxalase activities of human neutrophils during activation following treatment with opsonised zymosan.

Incubations contained $10^7$ neutrophils/ml in incubation buffer, pH 7.4 and 37°C, with and without 3.0 mg/ml opsonised zymosan. Key -○- Change in the activity of glyoxalase I; -●- change in the activity of glyoxalase II.

Data illustrated are from four experiments. Data points are mean ± s.e.m. Mean resting cell activities (one unit is the conversion of 1 μmol substrate to product per minute): glyoxalase I activity (mU/$10^6$ cells) - x = 9.11, s.e.m. = 0.77 (n=6); glyoxalase II (mU/$10^6$ cells) $\bar{x}$ = 0.94, s.e.m. = 0.07 (n=6).

Figure 2. Changes in reduced glutathione and S-D-lactoylglutathione in human neutrophils during activation following treatment with opsonised zymosan

Neutrophils ($10^7$/ml) were incubated with and without 3 mg/ml opsonised zymosan at 37°C in a shaking incubator. Samples were withdrawn at the time indicated and assays performed as described in the Methods section. ○, □ Resting cells, ●, ■ Cells activated with opsonised zymosan. Data are from four experiments. Data points are mean ± s.e.m.

The concentration of reduced glutathione rapidly decreases and the concentration of S-D-lactoylglutathione rapidly increases in neutrophils following treatment with opsonised zymosan (Figure 2). Within a sample of four donors, following incubation of neutrophils for 10 min with 3 mg/ml opsonised zymosan, the mean concentration of S-D-lactoylglutathione increased by ca. 100% of the resting cell concentration (Table 1). The demand for reduced glutathione in the synthesis of S-D-lactoylglutathione is expected to be a contributory factor in the concomitant fall in the concentration of reduced glutathione in the neutrophil.

## DISCUSSION

S-D-Lactoylglutathione has been postulated as an intermediate of the glyoxalase system for many years (1), yet its role in glutathione metabolism is not understood. Similarly, although the presence of glyoxalase activity in the neutrophil has been known for many years (25), its metabolic function in

the neutrophil (and elsewhere) is not fully understood (26). The changes in neutrophilic glyoxalase actvities induced by TPA (8), and the ability of the glyoxalase intermediate S-D-lactoylglutathione to stimulate microtubule assembly (5), indicate that the glyoxalase system may be involved in the regulation of microtuble assembly in the neutrophil.

The concentration of S-D-lactoylglutathione increases by ca. 100% during activation induced by zymosan. Recent reports suggest that during this period, there is only a small increase (0-25%) in the number of cytoplasmic microtubules but the mean microtubular length increases by ca. 100% (29,30). S-D-Lactoylglutathione may stabilise assembled microtubules and thereby increase mean microtubular length.

The importance of the presence of S-D-lactoylglutathione in the neutrophil may be recognised by considering that neutrophils maintain extraordinarily high levels of this metabolite, essentially fixing one equivalent of reduced glutathione, during the respiratory burst. Throughout this period, there is increased demand for reduced glutathione as part of the neutrophil self-protective antioxidant mechanisms, for example, as the cofactor required for the metabolism of hydrogen peroxide and hydroperoxides by glutathione peroxidase. The synthesis of S-D-lactoylglutathione appears to be an important feature of the response of the neutrophilic glutathione metabolism to functional activation induced by the zymosan stimulus, cf. (28).

When neutrophils are challenged with opsonised zymosan particles, they are functionally activated (a response to complement C3b). The particle is engulfed by phagocytosis, a membrane-bound superoxide-forming NADPH oxidase is activated, and lysosomal granules are projected into the phagosome (18-24). During activation, there is a marked increase in length of cytoplasmic microtubules (30). The mechanism of control of the neutrophilic microtubular network during this period is not fully understood. The phosphorylation of microtubule-associated proteins and post-assembly chemical modifications of microtubules may be involved (31,32). Our investigation suggests that the glyoxalase system, through the influence of S-D-lactoylglutathione, may be involved in the regulation of microtubule-assembly during the functional activation of neutrophils.

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