Mechanism and relevance of glutathione mutagenicity

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Summary

The ubiquitous tripeptide glutathione (GSH) has previously been shown to be mutagenic to Salmonella typhimurium TA100 when incubated with kidney subcellular fractions at physiological concentrations (Glatt et al., 1983). Here we report that the mutagenic effect of GSH can be inhibited by the use of the γ-glutamyl-transpeptidase (γ-GT) inhibitor anthglutin and by the metal chelators bathocuproine, EDTA and diethyldithiocarbamate. As the chelating agents did not inhibit γ-GT activity this suggested that the mechanism underlying the mutagenic effect of GSH was at least a two-step process, dependent upon the cleavage of GSH by γ-GT and the presence of either free transition metals or those contained in enzymes such as glutathione oxidase. As γ-GT is located on the outer surface of kidney tubule cells and is therefore exposed to relatively low concentrations of GSH, and the precise physiological control of levels of transition metals, this mechanism is unlikely to occur in vivo.

It has been reported by Glatt et al. (1983) that the physiologically important tripeptide glutathione (GSH) was mutagenic in the Salmonella reversion assay for mutagenicity using Salmonella typhimurium strain TA100. Cysteine was also found to be mutagenic, confirming previous work (Yamaguchi and Yamashita, 1981). Significant mutagenic effects were observed for GSH at concentrations of 5 mM and above using kidney, but not liver, postmitochondrial supernatant (S9). These concentrations, although high, are representative of intracellular physiological levels of GSH (Kosower and Kosower, 1978) whereas extracellular GSH concentrations are much lower, e.g. the level of GSH in rat plasma is in the order of 25 μM (Anderson and Meister, 1980).

Because of the relationship between carcinogenicity and mutagenicity (Ames and McCann, 1981), these results suggested that GSH, a compound ubiquitous in mammalian systems, may represent a carcinogenic hazard. In an attempt to clarify this we have studied the mechanism of GSH-mediated mutagenicity in TA100.

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Materials and methods

Reduced glutathione (GSH), oxidized glutathione (GSSG), horseradish peroxidase (HRP), γ-glutamyl-transpeptidase (γ-GT), bathocuproine sulfonate, EDTA, diethyldithiocarbamate, γ-glutamyl-3-carboxy-4-nitroanilide were obtained from Sigma Chemical Co., St. Louis, MO. 1-γ-L-Glutamyl-2-[2-carboxy-phenyl]hydrazine (anth glutatin) was a gift from Sankyuo Fermentation, Tokyo.

Subcellular fractions were prepared from the kidneys of male Sprague-Dawley rats by standard procedures (Ernster et al., 1962). A partially purified plasma membrane fraction was prepared from a kidney homogenate according to Coleman et al. (1967). The methods used allowed preparation of all fractions from the same kidney homogenate.

Mutagenicity tests using Salmonella typhimurium strain TA100 were performed as described in Maron and Ames (1983). Tests were initiated by mixing 0.4 ml of a neutralized solution of GSH (100 mM) with subcellular preparations and bacteria (2 × 10^6) in 2 ml of top agar containing 0.05 mM histidine/biotin solution. This was then poured onto plates containing minimal agar, incubated for 2 days in the dark at 37°C and the colonies reverting to histidine independence counted (Maron and Ames, 1983). Protein was determined according to Lowry et al. (1951).

γ-GT activity was measured as described in Persijn and van der Silk (1976) in the presence of partially purified plasma membrane (equivalent to approx. 4 μg protein). Inhibitors were preincubated with the kidney preparation for 2 min prior to addition of substrate. GSH and GSSG were determined by HPLC according to Reed et al. (1980). Glutathione oxidase activity of the partially purified plasma membrane preparation was determined during a 30-min incubation at 37°C with 5 mM GSH. Glutathionyl radicals were generated during HRP-catalyzed oxidation of p-phenetidine in the presence of 5 mM glutathione (Ross et al., 1984, 1985) using the following reaction conditions: p-phenetidine (0.05 mM), HRP (0.2 μg/ml), and hydrogen peroxide (0.5 mM). The production of glutathionyl radicals was verified by ESR spectroscopy (data not shown).

Results and discussion

The kidney S9-dependent mutagenicity of GSH (5–20 mM) was confirmed in strain TA100 (data not shown) and a concentration of 20 mM was chosen for subsequent studies. In agreement with Glatt et al. (1983) we found that liver S9 did not catalyze the mutagenic effect of GSH and oxidized glutathione (GSSG) was not mutagenic up to concentrations of 10 mM. The subcellular localization of the kidney enzymes which catalyzed the mutagenic effect of GSH is shown in Table 1. The enzymes responsible were concentrated in membrane-containing fractions and were richest in the partially purified plasma membrane preparation. Amongst the enzymes that are located in the kidney plasma membrane in high concentration are γ-glutamyl-transpeptidase (γ-GT) and glutathione oxidase (Jones et al., 1979; Ormstad et al., 1979; Tate et al., 1979; Tate and Orlando, 1979; Ormstad and Orrenius, 1980; Griffith and Tate, 1980; Ashkar et al., 1981). The latter is a copper-containing enzyme and can be inhibited by the use of metal-chelating agents (Ormstad et al., 1981), whereas the activity of γ-GT can be specifically inhibited by a variety of compounds, including

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Revertants/plate/mg protein</th>
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<tbody>
<tr>
<td>Postmicrosomal supernatant</td>
<td>nd^a</td>
</tr>
<tr>
<td>Postmitochondrial supernatant (S9)</td>
<td>135</td>
</tr>
<tr>
<td>Microsomes</td>
<td>419</td>
</tr>
<tr>
<td>Partially purified plasma membrane</td>
<td>1005</td>
</tr>
</tbody>
</table>

^a nd, not detectable.

Results are corrected for the mutagenic activity observed in control incubates either without kidney preparation or without GSH. Salmonella tester strain TA100 and 20 mM GSH were used in all experiments. Spontaneous reversion frequency = 35. Protein used per plate was: postmicrosomal supernatant, 2.5 mg; S9, 2.1 mg; microsomes, 0.5 mg; plasma membrane, 0.4 mg.
TABLE 2

EFFECT OF METAL-CHELATING AGENTS AND ANTHGLUTIN ON THE MUTAGENICITY OF GSH AND THE γ-GT ACTIVITY OF A PARTIALLY PURIFIED KIDNEY PLASMA MEMBRANE PREPARATION

<table>
<thead>
<tr>
<th>Additions (all at 1 mM)</th>
<th>(A) Inhibition of mutagenic activity(^a) of GSH (20 mM) (%)</th>
<th>(B) Inhibition of plasma membrane γ-GT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma membrane(^b)</td>
<td>S9(^c)</td>
</tr>
<tr>
<td>Anthglutin</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>Bathocuproine</td>
<td>100</td>
<td>37</td>
</tr>
<tr>
<td>EDTA</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>Diethyldithiocarbamate</td>
<td>100</td>
<td>79</td>
</tr>
</tbody>
</table>

\(^a\) Salmonella tester strain TA100 was used in all experiments. Spontaneous reversion frequency = 127.

\(^b\) Mutagenic activity of GSH (20 mM) — 1853 revertants/plate/mg protein corrected for control reversion frequency in the presence of GSH alone. 0.2 mg plasma membrane protein used per plate.

\(^c\) Mutagenic activity of GSH (20 mM) — 172 revertants/plate/mg protein corrected for control reversion frequency in the presence of GSH alone. 2.7 mg S9 protein used per plate.

Anthglutin (1-γ-L-glutamyl-2-[2-carboxyphenyl]-hydrazine (Minata, 1979).

The effect of the metal chelators bathocuproine, EDTA and diethyldithiocarbamate and the γ-GT inhibitor anthglutin on kidney plasma membrane- and S9-dependent, GSH-mediated mutagenicity is shown in Table 2A. All of these compounds totally inhibited the mutagenic activity of GSH with kidney plasma membrane fraction and inhibited to varying degrees with kidney S9. Only anthglutin, however, inhibited the γ-GT activity of the kidney plasma membrane fraction (Table 2B), purified γ-GT (Sigma) or kidney S9 (data not shown). Conversely, only the metal chelators inhibited the glutathione oxidase activity of the plasma membrane fraction (Table 3).

These results imply that the mechanism of mutagenicity of GSH when incubated with kidney subcellular fractions involves γ-GT-catalyzed cleavage as a critical event. As the chelating agents also inhibited the kidney plasma membrane-dependent mutagenic activity of GSH, but did not affect γ-GT activity, the mechanism must be at least a two-step process — one involving γ-GT and another requiring the activity of transition metals. The metal-chelating agents used will both chelate free metals and inhibit enzymes which depend on transition metals for their activity such as glutathione oxidase. Thus the second step in the mechanism underlying the induction of a GSH-dependent mutagenic effect in TA100 could presumably involve either free metals in solution or those contained in enzymes such as glutathione oxidase.

Our results do not enable us to unequivocally identify the mutagenic species derived from GSH but the γ-GT and metal dependency of the mutagenic activity allows us to propose some mechanisms consistent with the data. γ-GT catalyzed cleavage of glutathione to cysteinylglycine and metal-catalyzed autoxidation reactions of the latter may result in the production of cysteinylglycine radicals, other thiol-containing oxidation products and superoxide radicals (Misra, 1974; Hamed et al., 1983; Rowley and Halliwell, 1982), which may induce a genotoxic effect. Furthermore, cysteinylglycine may be hydrolyzed further to yield cysteine which may autoxidize to yield cysteine radicals. Autoxidation of thiols or the activity of glutathione oxidase can also generate hydrogen peroxide. This may then react with iron and copper in their reduced form, due to the high concentration of thiol present, in a Fenton reaction.

TABLE 3

EFFECT OF METAL-CHELATING AGENTS AND ANTHGLUTIN ON THE GSH OXIDASE ACTIVITY OF A PARTIALLY PURIFIED PLASMA MEMBRANE PREPARATION

<table>
<thead>
<tr>
<th>All additions at 1 mM</th>
<th>GSH oxidase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100(^a)</td>
</tr>
<tr>
<td>EDTA</td>
<td>23</td>
</tr>
<tr>
<td>Diethyldithiocarbamate</td>
<td>13</td>
</tr>
<tr>
<td>Bathocuproine</td>
<td>24</td>
</tr>
<tr>
<td>Anthglutin</td>
<td>&gt; 100</td>
</tr>
</tbody>
</table>

\(^a\) Equivalent to 59.5 nmoles/min/mg protein.

GSH oxidation rates in the absence of enzyme were approximately 29% of the control value.
generating hydroxyl radicals (Aust et al., 1985). The latter is a very reactive species which can damage DNA (Wilson, 1978). Thiyl radicals have also been proposed as mediators of the mutagenic effects of GSH (Glatt et al., 1983), but using enzymatic systems to generate glutathionyl radicals (see Materials and methods), we observed no GSH-dependent mutagenic activity. This does not rule out the possibility that cysteinylglycine or cysteine radicals may be mutagenic. Moreover, when glutathionyl radicals are produced extracellularly, they may be incapable of entering the cell and thus uptake of the parent thiol followed by subsequent intracellular reactions may be required for the mutagenic effect. The involvement of thiols, hydrogen peroxide and metal ions in reactions leading to the induction of a cytotoxic effect in E. coli K12 has previously been described (Berglin et al., 1982).

Whatever the identity of the ultimate mutagenic species it seems clear that the mechanism underlying the mutagenicity of GSH in the presence of kidney subcellular preparations involves γ-GT and metal-catalyzed reactions. Elucidation of this mechanism allows us to assess the potential in vivo hazard of the GSH-dependent mutagenicity observed in vitro. The enzymes γ-GT and glutathione oxidase are normally located on the outer surface of kidney cells (Meister and Tate, 1976; Lash and Jones, 1982; Lash et al., 1984) and are therefore exposed to only relatively low concentrations of GSH and do not come into contact with the much higher millimolar concentrations of GSH located within the cell. Furthermore, cellular control of the levels of free metals is carefully controlled by means of metal-binding proteins (Aust et al., 1985). On the basis of these results it can be concluded that the mutagenic effect of GSH observed in vitro is unlikely to represent an in vivo hazard.

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References


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