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CONCERTED ACTION OF REDUCED GLUTATHIONE AND SUPEROXIDE DISMUTASE IN PREVENTING REDOX CYCLING OF DIHYDROXYPYRIMIDINES, AND THEIR ROLE IN ANTIOXIDANT DEFENCE

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Dialuric Acid, the reduced form of the β-cell toxin alloxan, and the related fava bean derivatives divicine and isouramil, autoxidize rapidly in neutral solution by a radical mechanism. GSH promotes redox cycling of each compound, with concomitant GSH oxidation and H_2O_2 production. With superoxide dismutase present, there is a lag period in which little oxidation occurs, followed by rapid oxidation. GSH extends this lag and decreases the subsequent rate of oxidation, so that with superoxide dismutase and a sufficient excess of GSH, coupled oxidation of GSH and each pyrimidine is almost completely suppressed. This mechanism may be a means whereby GSH in combination with superoxide dismutase protects against the cytotoxic effects of these reactive pyrimidines. Superoxide dismutase may also protect cells against oxidative stress in other situations where GSH acts as a radical scavenger, and we propose that the concerted action of GSH and superoxide dismutase constitutes an important antioxidant defence.

KEY WORDS: Glutathione, superoxide dismutase, intracellular antioxidant defence, dialuric acid, alloxan, divicine, isouramil, autoxidation.

ABBREVIATIONS: G6PD; glucose-6-phosphate dehydrogenase: SOD; superoxide dismutase.

INTRODUCTION

Reduced glutathione (GSH) reacts with a wide variety of free radicals, and is considered to function biologically as a free radical scavenger and in the repair of radical-mediated biological damage.[1-3] However, reactions of GSH with radical species generate the thiyl radical ($GS^{\cdot}$), so GSH will be protective only if subsequent reactions of $GS^{\cdot}$ are benign.

The reactions of thiyl radicals have been characterized mainly by the use of radiolytic techniques.[4-7] In aerobic solution, $GS^{\cdot}$ can dimerize, react directly with O_2 to form a peroxyl radical (reaction (1)) or react with the thiolate ion and then with O_2 (reactions 2 and 3).

$$GS^{\cdot} + O_2 \rightleftharpoons GSOO^{\cdot} \quad (1)$$

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$$GS^{\cdot} + GS^{-} \rightleftharpoons GS^{\cdot}S^{-}G \quad (2)$$

$$GS^{\cdot}S^{-}G + O_2 \rightarrow GSSG + O_2^{-} \quad (3)$$

However, dimerization is a minor reaction, and reaction (1) is reversible,[6,7] so that at pH > 7 and with GSH > mM, reaction (2) predominates and the majority of $GS^{\cdot}$ gives rise to O_2^{-}.

Thus, radical scavenging by GSH initiates a sequence in which one radical ($GS^{\cdot}$) is replaced by another (O_2^{-}). This would be beneficial only if the O_2^{-} was broken down harmlessly. This is frequently not the case. As we shall describe, radical scavenging by GSH can result in a superoxide-dependent chain sequence in which GSH is oxidized to GSSG, and O_2 reduced to H_2O_2, in yields far in excess of the initial oxidizing event. SOD prevents such a sequence.

Physiologically, the intracellularly location of GSH means that it exists in association with SOD. This association should enable GSH to function as a free radical scavenger without concomitant oxidative stress to the cell. The combined action of GSH and SOD could, therefore, be an important intracellular antioxidant defence mechanism. In this presentation, we shall show that this mechanism operates to prevent autoxidation of the cytotoxic hydroxypyrimidines, dialuric acid, divicine and isouramil, and propose that it has wider application to protect cells against radical-mediated oxidative damage.

AUTOXIDATION OF DIALURIC ACID, DIVICINE AND ISOURAMIL

Dialuric acid (2,4,5,6-tetrahydroxypyrimidine) is the reduced form of the pancreatic β-cell toxin, alloxan. Divicine (2,4-diamino-5,6-dihydroxypyrimidine) and isouramil (4-amino-2,5,6-trihydroxypyrimidine) are the haemolytic agents produced by hydrolysis of the fava bean constituents, vicine and convicine. They can cause severe red cell haemolysis in glucose-6-phosphate dehydrogenase (G6PD) deficiency.

All three compounds autoxidize rapidly in neutral solution, via a free radical mechanism in which H_2O_2 is an end product.[8-10] Catalysis by trace metal ions generally contributes to the reaction, but with a chelator present to inhibit metal-catalysed oxidation (as in all the experiments discussed below) oxidation is still complete within a few minutes. SOD inhibits the initial reaction, but after a lag period rapid oxidation occurs,[10] as in Figure 1. The lag decreases with increasing pyrimidine concentration, and the reaction rate during the rapid oxidation phase may be faster than that in the absence of SOD.

By measuring both O_2 uptake and spectral changes of the pyrimidine solutions at different reactant concentrations, and carrying out kinetic analysis of the data, we have found[10] that autoxidation of all three pyrimidine can be explained by the following mechanism. Differences in individual rate constants account for differences in shape of the rate curves.

$$DH_2 + O_2 \rightarrow O_2^{-} + DH^{\cdot} + H^{+} \quad (4)$$

$$H^{+} + DH_2 + O_2^{-} \rightarrow DH^{\cdot} + H_2O_2 \quad (5)$$

$$DH^{\cdot} + O_2 \rightleftharpoons D + O_2^{-} + H^{+} \quad (6f,6r)$$

$$DH_2 + D \rightleftharpoons 2DH^{\cdot} \quad (7f, 7r)$$

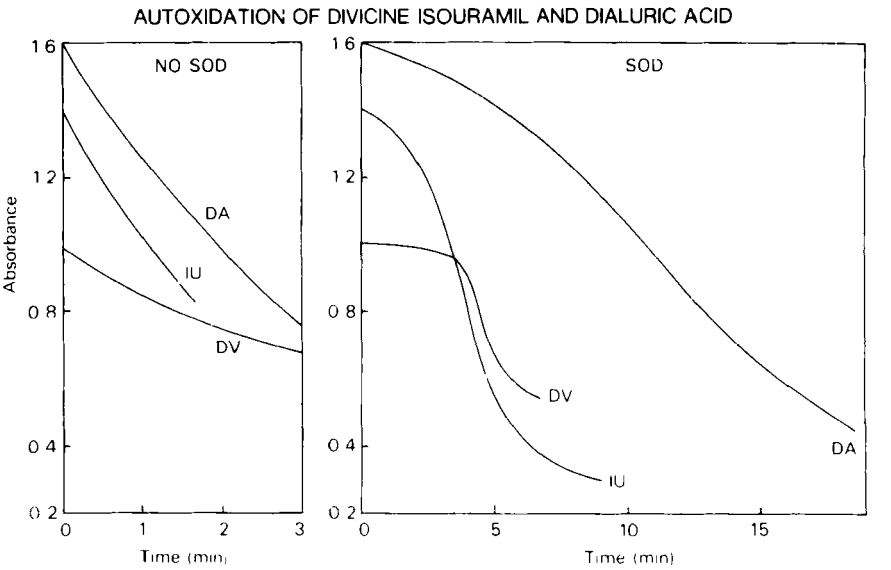

FIGURE 1 Time course for oxidation of dialuric acid (DA), divicine (DV) and isouramil (IU) in the presence and absence of SOD. Solutions in phosphate buffer pH 7.4 containing DTPA were incubated at 23°. Loss of each compound (100 μM), was followed spectrally at 273 nm (DA) or 280 nm (DV and IU), as in.[10]

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (8)$$

(DH_2, pyrimidine hydroquinone; D, quinone; $DH^{\cdot}$, semiquinone radical.)

Initiation by reaction (4) is slow, with $k_4 < 0.5\,M^{-1}s^{-1}$ at pH 7.4 and 23°C. Once initiated, oxidation proceeds by a chain consisting of reactions (5) and (6f), in which O_2^- and the pyrimidine radical are intermediates. Values for k_5 and k_{6f} range between 10^4 and $> 10^5\,M^{-1}s^{-1}$. SOD, by catalysing reaction (8), suppresses reaction (6f) and inhibits this chain. Oxidation then occurs by reactions (5) and (7f). The requirement for oxidized pyrimidine explains the lag seen with SOD, and the ability of SOD to displace equilibrium (6) to the right by inhibiting the reverse reaction explains why the maximum rate of oxidation can be faster in the presence of SOD.

Thus, the primary route for oxidation of dialuric acid, divicine and isouramil is a superoxide-dependent chain. However, even though SOD inhibits this sequence, oxidation is delayed only a few minutes under the conditions of Figure 1, and even less at 37° or with higher pyrimidine concentrations.[10] SOD alone, therefore, is unlikely to protect cells against autoxidation of the pyrimidines.

EFFECT OF GSH ON AUTOXIDATION

Addition of GSH to dialuric acid, divicine or isouramil decreases the net rate of pyrimidine loss but does not affect the rate of O_2 uptake.[11,12] However, whereas in the absence of GSH, O_2 uptake is equimolar with pyrimidine consumption, with GSH present O_2 uptake continues until either the O_2 or the GSH is all consumed. This indicates redox cycling of the pyrimidine, and a change in stoichiometry from

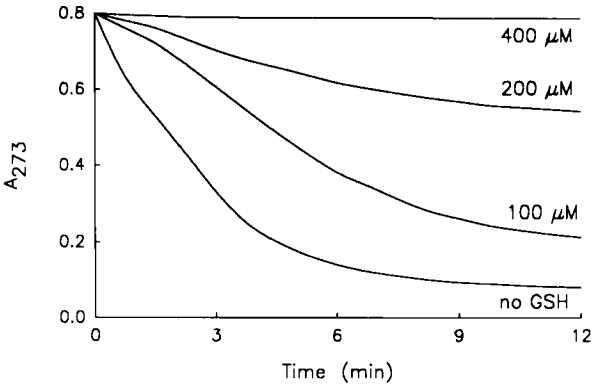

FIGURE 2 Effect of GSH on the time course of dialuric acid autoxidation in the presence of SOD. Solutions were incubated under the same conditions as in Figure 1, except at 37° and with 50 μM dialuric acid and 0, 100, 200 or 400 μM GSH. Data taken from.[11]

$$DH_2 + O_2 \rightarrow D + H_2O_2 \quad (9)$$

to

$$2GSH + O_2 \rightarrow GSSG + H_2O_2 \quad (10)$$

Consistent with this, all the GSH consumed is converted to GSSG.[11]

When GSH is added to the pyrimidines in the presence of SOD, it extends the lag phase and decreases the maximum rate of oxidation.[11,12] This is seen both when monitoring spectral changes (Figure 2) and O_2 uptake (Figure 3), and loss of thiol groups follows a similar time course. With a 10–20 fold excess of GSH over pyrimidine, there is no loss of pyrimidine, GSH oxidation or O_2 uptake for periods in excess of an hour. We conclude from these findings that:

1) GSH prevents autoxidation of dialuric acid, divicine and isouramil in the presence but not in the absence of SOD.

2) SOD inhibits redox cycling of the hydroxypyrimidines in the presence of GSH.

These observations are compatible with the mechanism described by reactions (4–8), with GSH scavenging the pyrimidine semiquinone ($DH^{\cdot}$) and being stoichiometrically oxidised to GSSG with concurrent $O_2^{\cdot-}$ generation. This enables superoxide-dependent chain oxidation to continue unabated. These requirements are met by reactions (11), (2) and (3).

$$DH^{\cdot} + GSH \rightleftharpoons DH_2 + GS^{\cdot} \quad (11)$$

Reaction (11) is probably reversible, but driven in a forward direction by $GS^{\cdot}$ reacting in (2). The lack of inhibition of this chain by GSH, plus the stoichiometric conversion of GSH to GSSG, indicates that reactions of $GS^{\cdot}$ that do not generate $O_2^{\cdot-}$ (e.g. dimerization or reaction (1) must be unimportant. In the presence of SOD, oxidation is dependent on the buildup of product (D) to react in (7). At high enough GSH concentrations, reaction (11) outcompetes (6) and prevents this from occurring. The superoxide-dependent chain is suppressed and oxidation is restricted to the very slow direct reaction between O_2 and the reduced pyrimidine.

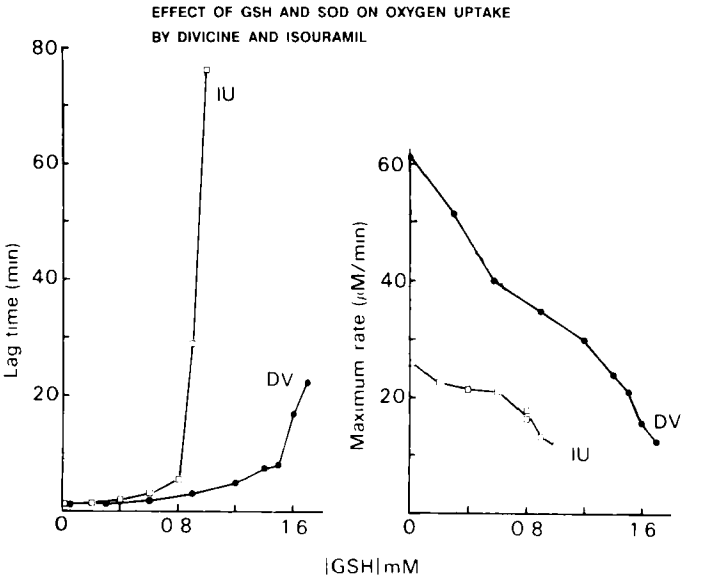

FIGURE 3 Effect of GSH on the lag time and maximum rate of oxygen uptake by solutions of divicine (DV) and isouramil (IU) (75 μM), incubated in an oxygen electrode chamber under the same conditions as in Figure 2. Results are taken from.[12]

CONCLUSIONS

These findings have implications for the cytotoxicity of dialuric acid and the favic pyrimidines. GSH is usually considered to protect against redox active drugs primarily by acting with glutathione peroxidase to remove H_2O_2, generated during redox cycling. The sensitivity of G6PD-deficient red cells to divicine and isouramil is usually explained on this basis. Our results suggest that GSH is also needed to act in concert with SOD to prevent redox cycling and the production of H_2O_2. Both mechanisms would be compromised in G6PD deficiency. Oxidation of the pyrimidines should also be able to occur in cells with low SOD levels. While this has not been documented for red cells, the low SOD levels reported for pancreatic β-cells[13] may be a factor in their susceptibility to alloxan/dialuric acid.

Neither GSH nor SOD alone can prevent autoxidation of the hydroxypyrimidines. In fact GSH enhances redox cycling and could exacerbate cell damage. It may not be coincidental, therefore, that physiologically, the two occur together. Hydroxypyrimidines are not the only compounds known to undergo superoxide-dependent chain oxidation. Many quinols, aromatic amines and thiols, catecholamines such as epinephrine and 6-hydroxydopamine, hydrazines and sugars that can form enediols behave in this way.[8,14–20] GSH is a good scavenger in these systems, and in several[19,20] has been shown to prolong O_2 uptake and conversion to H_2O_2.

We propose, therefore, that SOD functions to prevent chain oxidation of GSH in a wide range of radical-generating systems, and that the combination of GSH and SOD is an important intracellular antioxidant defence mechanism.

Protection by GSH and SOD could also be important in situations not involving autoxidizable compounds. A number of compounds such as haloalkanes, phenols and aromatic amines can be oxidised to their corresponding radicals e.g. by peroxidases or cytochrome P450.[21,22] Carbon- and oxygen-centred radicals are also formed when cell constituents are exposed to ionizing radiation. GSH scavenges these radicals,[1,21–23] regenerating the parent compounds, and often inhibiting peroxidative chains. This process could ameliorate the harmful effects of xenobiotics, and repair by GSH or other thiols is thought to be important in radiation protection. However, the $GS^{\cdot}$ formed by radical scavenging will act as a source of O_2^- so that chain oxidation of other O_2^- reactive compounds could still proceed. The effectiveness of GSH-mediated detoxication or radiation protection should, therefore, depend on the presence of SOD.

Finally, it appears that GSH itself can undergo O_2^--dependent chain oxidation. Several authors have investigated the reaction between O_2^- and GSH (reaction 12) or other thiols, with reported rate constants[24] varying between $< 10^3$ and 7×10^5 $M^{-1} s^{-1}$.

$$GSH + O_2^- + H^+ \rightarrow GS^{\cdot} + H_2O_2 \qquad (12)$$

A number of studies have shown that O_2^- can oxidise GSH under physiologically relevant condition, and in some cases O_2 uptake and disulphide formation indicative of a chain reaction have been measured.[4,23,25] While oxidation of a single GSH by O_2^- may not constitute on oxidative stress, occurrence of chain oxidation within a cell may be a different story. One reason why cells need SOD may be to prevent such GSH oxidation from occurring.

Acknowledgement

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