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Assessment of the relationship between the antimutagenic action of riboflavin and glutathione and the levels of antioxidant enzymes

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Abstract

In this study the role of antioxidant enzymes on the antimutagenic actions of riboflavin and reduced glutathione against mutagenic potentials of 4-nitroquinoline 1-oxide and mitomycin C have been investigated. For this purpose the activities of catalase and superoxide dismutase enzymes have been determined in *Salmonella typhimurium* TA102 and TA100 strains preincubated with different combinations of 4-nitroquinoline 1-oxide, mitomycin C, riboflavin and reduced glutathione for thirty minutes. Also in part of the same samples, the mutagenicity has been determined for each combination of chemicals by using Salmonella preincubation test. The correlation between the levels of antioxidant enzymes and mutagenicity and antimutagenicity has been investigated.

While riboflavin displayed a weakly antimutagenic effect on 4-nitroquinoline 1-oxide mutagenicity in TA102 and TA100 (0.25, 0.35 inhibition respectively), it did not have any effect on the strong mutagenicity of mitomycin C in both strains. Reduced glutathione, a well known antioxidant, had no antimutagenic effect against the mutagenicity of both compounds in TA102 and TA100 strains. The antioxidant enzymes, catalase and superoxide dismutase, seemed to have no direct effect on the antimutagenic action of riboflavin and mutagenic action of 4-nitroquinoline 1-oxide and mitomycin C because no change in the activities of catalase and superoxide dismutase was detected in relation to antimutagenicity of riboflavin and mutagenicity of 4-nitroquinoline 1-oxide and mitomycin C in both strains. It should be noted that many antimutagens have more than one mechanism of action and their effect depends on the mutagens being tested.

Keywords: Antimutagenicity; Antioxidant enzymes; Salmonella mutagenicity test

1. Introduction

Oxygen free radicals play a prominent role in tumor promotion. They are part of the oxidative metabolism of mutagens and, in addition, possess genotoxic properties [1]. An excess generation of free radicals and a deficient cellular antioxidant defense system may lead to a state of oxidative stress which may contribute to the development of cancer [2]. The mode of damage caused by oxidants is rather complicated. They can cause cell damage by affecting the cell membrane, the genetic material and various enzymatic reactions [3]. These oxidants are counteracted by enzyme systems such as catalase, superoxide dismutase (SOD) and glutathione peroxidase and by non-enzymatic antioxidant systems in the organism [4]. Therefore antioxidant enzymes have a great importance in antimutagenic activity.

This study aimed to monitor the levels of two enzymes of the antioxidant system, catalase (EC 1.11.1.6) and SOD (EC 1.15.1.1), and to relate them to the effects of two exogenous antimutagens, riboflavin and reduced glutathione (GSH). The test system was Salmonella preincubation test with *S. typhimurium* TA102 and TA100. The mutagens used were 4-nitroquinoline 1-oxide (4NQO) and mitomycin C (MMC). 4NQO is a well-known mutagen which undergoes redox recycling to generate superoxide [5], 4NQO can influence antioxidant mechanisms. MMC was used to check whether riboflavin and GSH have any effects on the action of mutagens which act by a non-oxidative mechanism. MMC is known as a cross-linking alkylating agent which inhibits DNA replication [6], and it has previously been used as a positive control of *S. typhimurium* TA102 by Gasiorowski et al. [7]. The results were evaluated in terms of the possible mechanism of antimutagenic action.

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Table 1
The results of salmonella mutagenicity test with *S. typhimurium TA*102 *Strain*

Mutagen	Antimutagen	Concentration μmol/L	Number of revertants/plate[a]
Control[b]			292 ± 45
4NQO[c]		26	597 ± 63
MMC[c]		15	3884 ± 270
	Riboflavin	265	294 ± 41
4NQO	Riboflavin[d]	26 + 265	446 ± 61
MMC	Riboflavin	15 + 265	3360 ± 345
	GSH	1000	326 ± 59
4NQO	GSH	26 + 1000	690 ± 38
MMC	GSH	15 + 1000	4352 ± 439

[a] Each value is the mean ± S.D. of three replicates from each of three separate Salmonella preincubation tests of different combination of the chemicals.
[b] The average number of revertants, 292 ± 45, is the mean of fifty seven plaques.
[c] Mutagen. A compound was classified as a mutagen if there was at least a 2-fold elevation in frequency of spontaneous revertants [9 (p. 74)].
[d] A weak antimutagenic effect. A compound was classified as a weak antimutagen if the % inhibition of mutagenicity was between 0.20 and 0.40 [20 (p. 223)].

Table 2
The results of salmonella mutagenicity test with *S. typhimurium TA*100 *Strain*

Mutagen	Antimutagen	Concentration μmol/L	Number of revertants/plate[a]
Control[b]			143 ± 38
4NQO[c]		26	370 ± 117
MMC		15	25 ± 6
	Riboflavin	265	115 ± 26
4NQO	Riboflavin[d]	26 + 265	238 ± 31
MMC	Riboflavin	15 + 265	27 ± 3
	GSH	1000	155 ± 55
4NQO	GSH	26 + 1000	414 ± 156
MMC	GSH	15 + 1000	25 ± 19

[a] Each value is the mean ± S.D. of three replicates from each of three separate Salmonella preincubation tests of different combination of the chemicals.
[b] The average number of revertants, 143 ± 38, is the mean of forty one plaques.
[c] Mutagen. A compound was classified as a mutagen if there was at least a 2-fold elevation in frequency of spontaneous revertants [9 (p. 74)].
[d] A weak antimutagenic effect. A compound was classified as a weak antimutagen if the % inhibition of mutagenicity was between 0.20 and 0.40 [20 (p. 223)].

2. Materials and methods

The chemicals were obtained commercially from the following sources : 4NQO (Purity: >0.97), MMC and GSH (Purity: 0.98–0.99) from (Sigma Chemical Co., St. Louis, U.S.A.); Riboflavin (Purity: >0.99) from (Merck-Schuchardt, Darmstadt, F.R.G.). Compounds to be tested were dissolved in DMSO (4NQO, MMC) or in water (riboflavin, GSH).

Salmonella typhimurium TA102 (*his* G 428 (pAQ1) pKM101 rfa) and TA100 (*his* G 46 pKM101 rfa ΔuvrB) were kindly provided by Dr. Bruce Ames (University of California, Berkeley Ca., U.S.A.). The characteristics of the strain was confirmed by the procedure of Maron and Ames [8].

The amounts of test compounds to be used in the Salmonella mutagenicity test were selected with *S. typhimurium* TA102 based on the basis of preliminary cytotoxicity assays [9] and the same concentration also used with TA100.

The genotoxicity assay was performed on the *S. typhimurium* TA102 and TA100 strains according to the protocol of Yahagi et al. [10] as described by Gomes et al. [11]. In the protocol the experimental procedure adopted was the preincubation method. Briefly, 45 ml of the tester strain ($1–2 \times 10^{12}$ cells/L, determined on the basis of A_{600} values of the fresh cultures) were centrifuged at 3500 rpm for 10 min in a Beckman L-2 refrigerated centrifuge. The pelletted cells were resuspended in 0.2 mol/L phosphate buffer, pH 7.4 and divided into 9 tubes (5 ml suspension each) supplemented with: (1) DMSO 100 μl, (2) 26 μmol/L 4NQO, (3)

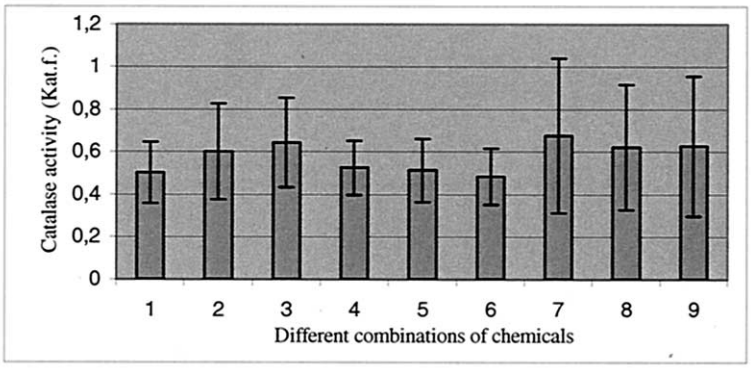

Fig. 1. Catalase activity in *S. typhimurium* TA102. (1) Control (DMSO); (2) 4NQO; (3) MMC; (4) Riboflavin; (5) 4NQO + Riboflavin; (6) MMC + Riboflavin; (7) GSH; (8) 4NQO + GSH; (9) MMC + GSH. Each value is the mean ± S.D. of three replicates from each of three separate experiments. There is no significant difference between control and groups ($p > 0.05$, ANOVA).

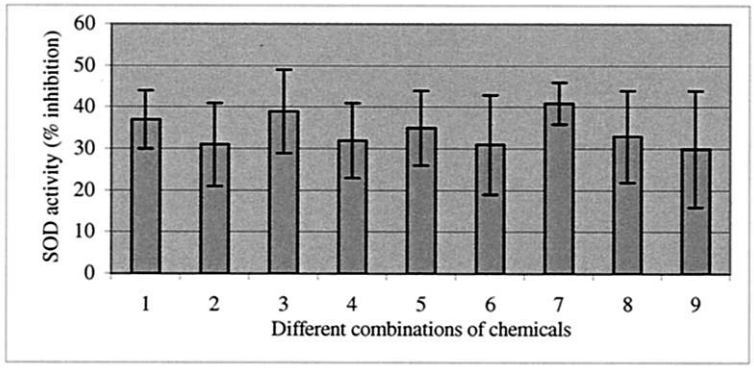

Fig. 2. SOD activity in *S. typhimurium* TA102. (1) Control (DMSO); (2) 4NQO; (3) MMC; (4) Riboflavin; (5) 4NQO + Riboflavin; (6) MMC + Riboflavin; (7) GSH; (8) 4NQO + GSH; (9) MMC + GSH. Each value is the mean ± S.D. of three replicates from each of three separate experiments. There is no significant difference between control and groups ($p > 0.05$, ANOVA).

15 μmol/L MMC, (4) 265 μmol/L riboflavin, (5) 26 μmol/L 4NQO + 265 μmol/L riboflavin, (6) 15 μmol/L MMC + 265 μmol/L riboflavin, (7) 1000 μmol/L GSH, (8) 26 μmol/L 4NQO + 1000 μmol/L GSH, (9) 15 μmol/L MMC + 1000 μmol/L GSH. The tubes were incubated for 30 min at 37°C. Following the incubation the cells were harvested, washed free of the chemicals and resuspended in the 5 ml of the buffer. A 100 μl of the aliquot was used in the mutagenicity assay. The remainder of the samples was sonicated and then assayed for catalase and SOD activity. A 100 μl of sample was used in each enzyme assay. The Salmonella mutation assay was carried out by adding the 100 μl aliquot to 2 ml top agar and pouring the mixture onto minimal glucose plates. The plates were incubated at 37°C for 48 h, and the number of mutant colonies was scored.

Catalase activity was measured by the method of Cohen et al. [12]. Under the conditions described the decomposition of H_2O_2 by catalase follows first-order kinetics as given by the following equation

$$k = \log(S_0/S_3) \times 2.3/t$$

where k is the first order reaction rate constant, t is the time interval over which the reaction is measured (3 min), S_0 is the substrate concentration at zero time and S_3 is the substrate concentration at 3 min. [To obtain S_0, subtract the absorbance of the reaction system blanks (B) from the spectrophotometric standard (St). To obtain S_3, subtract the absorbance of the reaction samples (A) from (St).]

The level of catalase is finally expressed in terms of Kat.f. with the use of the following equation

$$\text{Kat.f.} = 1/2.3 \times k/A_{600}$$

indexing enzyme activity to cell number.

SOD activity was measured by the method of Paoletti and Mocali [13] which monitors NADH oxidation. In this study % inhibition of NADH oxidation was used as the index of SOD activity. The source of the enzymes was equal in all tubes in the enzyme assays. % inhibition of NADH oxidation was calculated according to the following equation

$$\%\ \text{inhibition} = 100 - (\text{sample rate/control rate}) \times 100$$

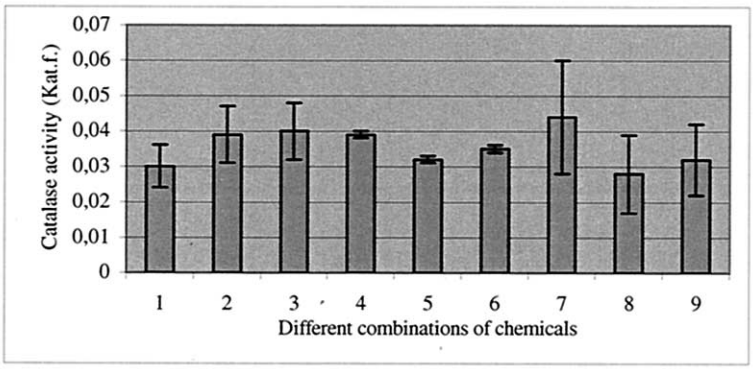

Fig. 3. Catalase activity in *S. typhimurium* TA100. (1) Control (DMSO); (2) 4NQO; (3) MMC; (4) Riboflavin; (5) 4NQO + Riboflavin; (6) MMC + Riboflavin; (7) GSH; (8) 4NQO + GSH; (9) MMC + GSH. Each value is the mean ± S.D. of three replicates from each of three separate experiments. There is no significant difference between control and groups ($p > 0.05$, ANOVA).

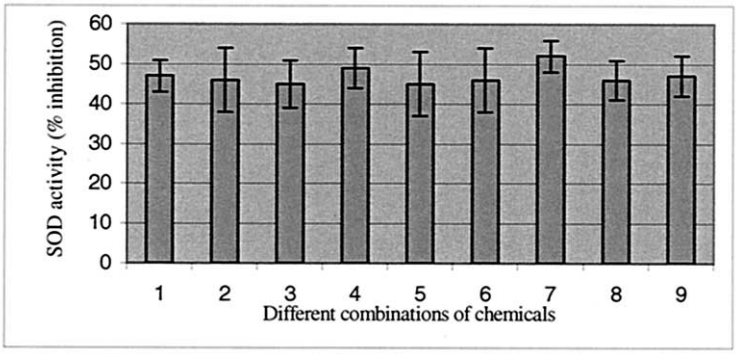

Fig. 4. SOD activity in *S. typhimurium* TA100. (1) Control (DMSO); (2) 4NQO; (3) MMC; (4) Riboflavin; (5) 4NQO + Riboflavin; (6) MMC + Riboflavin; (7) GSH; (8) 4NQO + GSH; (9) MMC + GSH. Each value is the mean ± S.D. of three replicates from each of three separate experiments. There is no significant difference between control and groups ($p > 0.05$, ANOVA).

3. Results and discussion

Table 1 and 2 show the results of Salmonella mutagenicity test with *S. typhimurium* TA102 and TA100 strains treated with various combinations of chemicals. Figs. 1 and 2 show the levels of catalase and SOD activity before and after treatment of the bacteria with mutagens and antimutagens, either single or in combination in *S. typhimurium* TA102, Figs. 3 and 4 show the levels of the same enzymes in TA100 strain.

Riboflavin showed a weak antimutagenic effect on the mutagenicity of 4NQO in both strains (0.25 in TA102 and 0.35 in TA100). Flavins were found to be active against mutagens such as 4NQO, AFB1, the condensation of cigarette smoke, B(a)P, UV irradiation and B(a)P-7,8-diol epoxide with Salmonella/microsome test system [14]. In spite of the observed antimutagenic potential, treatment with riboflavin, alone or in combination with 4NQO caused no change in the levels of catalase or SOD activity in both strains. Thus, it appears that the antimutagenic effect of this compound does not arise from a modification of the cellular antioxidant defense system. It could be that the observed effect is due, at least in part, to a direct scavenging action of the flavin on the reactive oxygen species generated by 4NQO. Riboflavin had no effect on the strong mutagenicty of MMC or on the levels of catalase and SOD in the presence of MMC. Riboflavin which acts as an electron acceptor was ineffective on MMC in *S. typhimurium* TA102 and TA100 which is a cross-linking alkylating agent.

GSH used as an antimutagen in our study is mainly a chemopreventive agent against mutagens and carcinogens, because it is capable of reacting directly with electrophiles [15]. GSH did not reduce the mutagenic effects of 4NQO and MMC on *S. typhimurium* TA102 and on TA100. Rather, it increased partly the mutagenicity of these chemicals. Possible explanations for the increase can be an increase in xenobiotic metabolism or the conversion of GSH metabolism to oxidative pathway due to the high level of γ-glutamyl transpeptidase (GGT) and by allowing formation of reactive oxygen derivatives and radicals [16]. When GSH was used either alone or with 4NQO and MMC, the enzyme activities did not reflect any influence of the treatments that was statistically significant in both strains.

It is known that the application methods of mutagens and antimutagens influence the results [17,18]. Therefore to make a definite evaluation about the contributions of chemicals and enzymes, pre- and post-treatment of antimutagens must be studied as emphasized by Balansky [19].

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