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Effect of α -Keto Acids Against H_2O_2 - and NaN_3 -Induced Mutagenesis in Different Strains of *Salmonella Typhimurium*

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Abstract: The protective effects of α -keto acids on the mutagenicity of H_2O_2 and NaN_3 was investigated in *Salmonella typhimurium* TA 98, TA 100, TA 102. H_2O_2 (340 μ g/plate) was mutagenic to the strains TA 98 and TA 102 while NaN_3 (1.5 μ g/plate) was mutagenic only to TA100. The extents of protective effect of pyruvate (220-440 μ g/plate) and α -keto glutarate (335-600 μ g/plate) against H_2O_2 were observed to be 37%- 65% in TA 98 and TA 102. α -keto acids were ineffective against NaN_3 induced mutagenicity in TA 100. H_2O_2 (340 μ g/plate), in the presence of NaN_3 (1.5 μ g/plate) and α -keto acids (330-500 μ g/plate), was found to be nonmutagenic to the strain TA102. This cross-adaptive response might have been due to the intactness of the DNA repair system in TA 102. The above combination of H_2O_2 , NaN_3 and α -keto acids were found to be cytotoxic in TA 98 and TA 100, possibly because these strains carry uvr B mutation causing defect in their DNA repair system.

Key Words: α -keto acids, H_2O_2 , NaN_3 , Ames test system, mutagenicity, antimutagenicity

Salmonella Typhimurium'un Farklı Suşlarında H_2O_2 ve NaN_3 ile İndüklenmiş Mutasyonlara Karşı α -Keto Asitlerin Etkisi

Özet: *Salmonella typhimurium* TA98, TA100 ve TA102 suşlarında α -keto asitlerin, H_2O_2 ve NaN_3 'nin mutajenitelerine karşı koruyucu etkileri araştırıldı. H_2O_2 (340 μ g/plak), hem TA98 hem de TA102 suşunda mutajenik iken, NaN_3 (1.5 μ g/plak) sadece TA100 suşunda mutajeniktir. Pürüvat (220-440 μ g/plak) ve α -ketoglutarat'ın (335-600 μ g/plak) H_2O_2 'ye karşı koruyucu etkileri, %37- %65 olarak bulundu. α -keto asitlerin, TA100 suşunda NaN_3 tarafından indüklenen mutajenesise karşı koruyucu etkilerinin olmadığı saptandı. TA102 suşunda, NaN_3 (1.5 μ g/plak) ve α -keto asitlerin (330-500 μ g/plak) varlığında, H_2O_2 'nin (340 μ g/plak) mutajenik etki göstermediği bulundu. Bu çapraz-adaptif cevap, TA102 suşunda DNA onarım sisteminin sağlam olmasından kaynaklanmış olabilir. TA98 ve TA100 suşlarında ise, H_2O_2 , NaN_3 ve α -keto asitlerin yüksek konsantrasyonlarındaki kombinasyonlarının sitotoksik etki gösterdikleri bulundu. Çünkü bu suşlar, DNA onarım sistemlerinde hasara sebep olan uvr B mutasyonu taşımaktadırlar.

Anahtar Sözcükler: α -keto asitler , H_2O_2 , NaN_3 , Ames test sistemi, mutajenite, antimutajenite

Introduction

The Ames test system is widely used to detect the mutagenicity and antimutagenicity of various chemicals. The test measures back-mutation in several constructed mutants of *Salmonella typhimurium* (1, 2). Hydrogen peroxide (H_2O_2) readily diffuses into bacterial cells and induce oxidative DNA damage (3, 4). Oxidative damage to DNA is mutagenic and thus considered to play a role in carcinogenesis (5, 6). α -Keto acids have been implicated as antioxidants reacting with H_2O_2 and concomitantly leading to its decomposition (7- 9). There is renewal of interest in H_2O_2 -mediated α -keto acid oxidation due to the consideration regarding these chemicals to be useful

from therapeutic point of view in several disease states from cancer to ageing (10-13). Hydrogen peroxide, organic peroxides and hydroperoxides are frequently used industrial chemicals, e.g. as a source of free radicals in the plastic and rubber industry, as bleaching agents for foods and paper pulps, in the production of textiles, cosmetics and pharmaceuticals, and as chemical intermediates (14). Sodium azide (NaN_3) is a highly reactive nucleophilic agent known as a classical base-substitution mutagen, causing DNA damage in *S. typhimurium* strain TA 100 (15), it is also reported as singlet oxygen scavenger and an inhibitor of catalase (16, 17). The main agent through which azide

mutagenesis occurs has not been determined yet. Possible mechanisms such as interaction with DNA which could lead to genotoxicity, mutagenicity and carcinogenicity by this class of compounds have been proposed (18).

H_2O_2 was shown to be mutagenic in a number of *S. typhimurium* strains by Salmonella mutagenicity test (19). In the present study Ames tester strains TA 98, TA 100, TA 102 were examined for H_2O_2 induced mutagenesis. The TA 102 strain has a unique sensitivity for reversion by chemical oxidants (20). The tester strains TA 98 and TA 102 which were found to be mutagenic for 340 μ g/plate H_2O_2 were further used for the investigation of the antimutagenic activities of α -keto acids, pyruvate and α -ketoglutarate. Contrary to α -keto acids that decompose H_2O_2 , sodium azide prevents degradation of H_2O_2 by several mechanisms (21).

The aim of this study was to test the effect of α -keto acids on H_2O_2 and NaN_3 induced mutagenesis in *S. typhimurium* TA mutant strains and discuss the effects leading to opposite results.

Materials and Methods

Chemicals and tester strains

Chemicals and their sources used were as the followings: Pyruvate, α -ketoglutarate, sodium azide, daunomycin and D-biotin (Sigma Chemical Co., St. Louis, USA), hydrogen peroxid (Merck-Schunck, Darmstadt, F.R.G.), L-histidine-HCl monohydrate (BDH), Bacto agar (Difco) and Oxoid nutrient broth no:2 (Oxoid).

S. typhimurium TA 98 (his D 3052, rfa, Δ uvr B, pKM 101), TA 100 (his G 46, rfa, Δ uvr B, pKM 101) and TA 102 (his G 8476, rfa, pAQ1/pKM101) were kindly provided by Dr. Bruce Ames (University of California, Berkeley Ca. U.S.A.) All strains were stored at -80° C and were routinely checked to insure the presence of appropriate genetic markers and spontaneous reversion patterns. Overnight growth was initiated with inoculation from master plate into Oxoid-nutrient broth no:2. Following overnight growth, all tester strains were diluted into the same culture and grown with shaking at 37° C (approximately 5 hours). When cultures reach a

density of 0.300 OD at 650 nm ($1-2 \times 10^9$ cells/ml), they were used in the mutagenicity experiments (1, 22).

Cytotoxicity testing

The amounts of test compounds to be used in the mutation assays were selected in cytotoxicity assay. Fully grown cultures of the strains were diluted 10^6 fold in saline. 0.1 ml of a diluted culture was added to 2 ml top agar, together with a different concentration of tested chemical. α -keto acids and H_2O_2 were dissolved in sterilized water and deionized water, respectively. The top agar was poured onto nutrient agar plates and assesment of cytotoxicity was made after 24 h incubation at 37° C (23).

Mutagenicity testing

In Salmonella/microsome test system, data are interpreted on the basis of a consistent doubling of the spontaneous reversion frequency confirmed by a dose-response relationship. Where the number of induced revertants is less than twice the spontaneous rate but a reproducible dose-related increase in revertants is detected, this is also interpreted as a positive response (23).

Antimutagenicity testing

The antimutagenicity testing consists of combining 0.1 ml from a culture of the tester strain, 0.1 ml of the test samples, 0.1 ml of positive mutagen in soft agar which was poured to on a minimal agar plate. After incubation at 37° C for 48-72 h the revertant colonies were counted to determine the inhibitory effects, expressed as the inhibition rate. The tested substances were considered to possess antimutagenic activity which was expressed with the positive rate: Antimutagenic Activity % = $(A-B)/A \times 100$, where A is positive mutagen revertant colonies, B is revertant colonies after adding the test sample (24).

Statistical Analysis

The analysis of variance was used to compare the experiments and Dunnett's method was used to validate the mutagenic action of the different concentrations as compared to the control in *S. typhimurium* (TA 98, TA100, TA 102) strains (25).

Results and Discussion

Mutagenicity of H₂O₂

H₂O₂ (340 µg/plate) was found to be mutagenic for *S. typhimurium* TA98 and TA 102 ($P < 0.05$) but was nonmutagenic for TA 100 in the absence of S9 mix ($P > 0.05$). The number of spontaneous revertant colonies for *S. typhimurium* TA 98, TA 100 and TA 102 were evaluated statistically and found as 23 ± 7 , 151 ± 19 , 274 ± 14 respectively. The highest mutagenic response was found in TA 102 (Table 1). The *S. typhimurium* TA 98 strain is used for the detection of frame-shift mutations, TA 100 and TA 102 strains are used for the detection of base-pair substitutions (1). Reports on the toxicity of H₂O₂ to cells are variable. The variability can be accounted for both by the activity of H₂O₂ removing enzymes and by the rate of conversion of H₂O₂ into more highly reactive radicals. The reactive oxygen species cause base-pair substitutions (26). For this reason, H₂O₂ is

mutagenic for TA 98 by a different mechanism. In fact TA 98 and TA 100 strains are not absolutely specific for base-pair substitution or frame-shift mutation respectively when compared to TA 1535 and TA 1538 (27).

Effect of α -keto acids on H₂O₂ mutagenesis:

The noncytotoxic concentration of H₂O₂ (50-340 µg/plate), pyruvate (55-440 µg/plate) and α -ketoglutarate (80-600 µg/plate) for the tester strains *S. typhimurium*

TA 98, TA 100 and TA 102 were determined and subsequently these concentrations were used throughout the experiments. In this study no significant increase in the number of his revertant colonies could be observed with the tester strain *S. typhimurium* TA 98, TA 100 and TA 102, whatever the concentration of pyruvate and α -ketoglutarate (Table 1). Pyruvate (55-110 µg/plate) and α -ketoglutarate (80-160 µg/plate) was ineffective for

Table 1. The mutagenic potentials of H₂O₂, NaN₃ and α -keto acids in Salmonella tester strains.¹

Compound	Dose (µg/plate)	Revertant Colony Numbers		
		TA 98 Mean±SD	TA 100 Mean±SD	TA 102 Mean±SD
Control	0	23 ± 7	151 ± 19	274 ± 1
H ₂ O ₂	340	46 ± 5	148 ± 31	705 ± 74
	100	29 ± 6	105 ± 12	370 ± 130
	50	25 ± 6	100 ± 11	255 ± 17
NaN ₃	1.5	31 ± 2	690 ± 80	265 ± 55
Pyruvate	55	24 ± 3.5	131 ± 5.9	267 ± 31
	110	22 ± 5.7	132 ± 8.5	316 ± 41
	220	25 ± 3.8	128 ± 7.3	288 ± 38
	330	22 ± 3.4	146 ± 28	224 ± 23
	440	20 ± 6.1	137 ± 16	225 ± 24
α -Ketoglutarate	80	27 ± 5	114 ± 12	276 ± 35
	160	26 ± 2	125 ± 12	301 ± 41
	335	23 ± 4	123 ± 22	296 ± 49
	500	26 ± 5	138 ± 25	238 ± 13
	600	23 ± 4	137 ± 24	240 ± 25
Positive Control				
Sodium azide (NaN ₃)	1.5	31 ± 2	690 ± 80	265 ± 55
Daunomycin	6	837 ± 127	-	1018 ± 114

¹ The results are means of 4 separate experiments with 3 plates each.

H_2O_2 mutagenesis in TA 98 and TA 102. Increasing concentrations of pyruvate (up to 440 μ g/plate) and α -ketoglutarate (up to 600 μ g/plate) decreased the H_2O_2 mutagenicity in *S. typhimurium* TA 98 and TA 102 (Table 2). The highest antimutagenic effect was observed in *S. typhimurium* TA 102 strain at 330 μ g/plate pyruvate (% 65) (Table 2).

α -keto acids nonenzymatically reduce H_2O_2 to water and decarboxylate 1-C carbon (7, 8). We observed the protective role of pyruvate (220-440 μ g/plate) and α -ketoglutarate (335-600 μ g/plate) in H_2O_2 induced mutagenicity in *S. typhimurium* TA 98 and TA 102 strains. Both α -keto-acids were nearly equally protective. Since *S. typhimurium* TA 102 showed the highest mutagenic response for H_2O_2 mutagenicity, the highest antimutagenic effect was observed in *S. typhimurium* TA 102.

Effect of α -keto acids on NaN_3 mutagenesis:

NaN_3 (1.5 μ g/plate) showed just the opposite effect of H_2O_2 induced mutagenicity in the tester strains used. It is a positive mutagen for TA 100 ($P < 0.05$) but nonmutagenic for *S. typhimurium* TA 98 and TA 102 ($P > 0.05$). Addition of pyruvate or α -ketoglutarate (at any concentration in TA 100 strain) did not change the sodium azide mutagenicity (Table 2). In previous work, it has been stated that sodium azide protects degradation of H_2O_2 (17).

Inorganic azide mutagenicity is mediated through a metabolically synthesized organic azide (28). Our data suggests that *S. typhimurium* TA 98 and TA 102 appear to be incapable of converting azide into a mutagenic intermediate in appreciable quantities, but TA 100 strain can.

Table 2. The antimutagenic effects of α -keto acids on NaN_3 and H_2O_2 induced mutagenesis in Salmonella tester strains.¹

Compound	Dose (μ g/plate)	Revertant Colony Numbers			Antimutagenic Activity (%)		
		TA 98 Mean \pm SD	TA 100 Mean \pm SD	TA 102 Mean \pm SD	TA 98 Mean \pm SD	TA 100 Mean \pm SD	TA 102 Mean \pm SD
H_2O_2 + Pyruvate	340 + 55	51 \pm 4	157 \pm 52	688 \pm 43	-10	-6	2
	340 + 110	52 \pm 8	171 \pm 1	627 \pm 18	-13	-15	-11
	340 + 220	29 \pm 6	169 \pm 14	315 \pm 29	37	-14	53
	340 + 330	28 \pm 2	156 \pm 12	248 \pm 42	39	-5	65
	340 + 440	26 \pm 3	153 \pm 8	338 \pm 24	44	-3	52
H_2O_2 + α -ketoglutarate	340 + 80	49 \pm 2	187 \pm 89	729 \pm 59	-7	-16	-3
	340 + 160	44 \pm 2	174 \pm 53	614 \pm 73	-7	-17	-3
	340 + 335	30 \pm 12	147 \pm 44	484 \pm 75	35	-1	31
	340 + 500	31 \pm 10	129 \pm 35	392 \pm 46	35	0	43
	340 + 600	24 \pm 10	141 \pm 36	328 \pm 72	48	-12	53
NaN_3 + Pyruvate	1.5 + 55	-	716 \pm 75	-	-	-3	-
	1.5 + 110	-	664 \pm 79	-	-	4	-
	1.5 + 220	-	713 \pm 103	-	-	-3	-
	1.5 + 330	-	650 \pm 53	-	-	6	-
	1.5 + 440	-	706 \pm 88	-	-	-2	-
NaN_3 + α -ketoglutarate	1.5 + 80	-	667 \pm 20	-	-	4	-
	1.5 + 160	-	693 \pm 43	-	-	0	-
	1.5 + 335	-	701 \pm 1	-	-	-2	-
	1.5 + 500	-	645 \pm 42	-	-	7	-
	1.5 + 600	-	695 \pm 81	-	-	0	-

¹ The results are means of 4 separate experiments with 3 plates each.

Effect of α -keto acids on NaN_3 - H_2O_2 mutagenicity:

NaN_3 (1.5 $\mu\text{g}/\text{plate}$) / H_2O_2 (340 $\mu\text{g}/\text{plate}$) or NaN_3 (1.5 $\mu\text{g}/\text{plate}$) / H_2O_2 (340 $\mu\text{g}/\text{plate}$) / α -keto acid (330-500 $\mu\text{g}/\text{plate}$) systems are cytotoxic in *S. typhimurium* TA 98 and TA 100 strains. Lowering the H_2O_2 concentration removes the cytotoxic effect of these systems in TA 98 and TA 100 (Table 3). NaN_3 inhibits both catalase and peroxidases through azidyl free radicals leading to increased H_2O_2 concentrations (21, 29, 30).

In TA 102 the above systems are noncytotoxic at any concentration. This strain has A-T base-pairs at the site for reversion and oxidative mutagens better than the other strains. In addition the DNA repair system is intact in TA 102 (20). There is no mutagenic effect of H_2O_2 in the presence of NaN_3 . H_2O_2 (340 $\mu\text{g}/\text{plate}$) is mutagenic in TA 102. In the presence of NaN_3 , the mutagenic effect of H_2O_2 is removed. This cross-adaptive response may be to the activation of DNA repair system by these chemicals upon H_2O_2 induced oxidative stress. DNA repair system is more important from catalase activity (31).

α -keto acids have no effect on NaN_3 mutagenicity, but combination of NaN_3 (1.5 $\mu\text{g}/\text{plate}$) + α -keto acids (330-600 $\mu\text{g}/\text{plate}$) + H_2O_2 (50 $\mu\text{g}/\text{plate}$) reduced NaN_3 mutagenicity by 13-24 % in *S. typhimurium* TA 100,

because NaN_3 is mutagenic only in TA 100, H_2O_2 at concentration 340 $\mu\text{g}/\text{plate}$ is cytotoxic in *S. typhimurium* TA 98 and TA 100 (Table 3). At the same concentration H_2O_2 is not cytotoxic in TA 102. Moreover, mutagenic activity of H_2O_2 (340 $\mu\text{g}/\text{plate}$) decrease by 54-63 % in the presence of NaN_3 (1.5 $\mu\text{g}/\text{plate}$). The results show that strains carrying DNA repair system challenge H_2O_2 cytotoxicity.

It is well known that H_2O_2 causes oxidative stress in the form of damaged purines and pyrimidines. Intact repair system in TA 102 may induce glycosylase which remove these oxidatively damaged purines and pyrimidines (26, 32).

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Table 3. Combined mutagenic activity of NaN_3 + H_2O_2 and NaN_3 + H_2O_2 + α -keto acids.¹

Compound	Dose ($\mu\text{g}/\text{plate}$)	Revertant Colony Numbers		
		TA 98 Mean+SD	TA 100 Mean+SD	TA 102 Mean+SD
Control	0	23 \pm 7	151 \pm 19	274 \pm 1
NaN_3 + H_2O_2	1.5 + 340	*cyt.	cyt.	494 \pm 73
	1.5 + 100	25 \pm 7	527 \pm 93	471 \pm 57
	1.5 + 50	23 \pm 3	534 \pm 78	218 \pm 17
NaN_3 + H_2O_2 + Pyruvate	1.5 + 340 + 330	cyt.	cyt.	233 \pm 30
	1.5 + 50 + 330	27 \pm 5	466 \pm 61	297 \pm 44
	1.5 + 340 + 440	cyt.	cyt.	234 \pm 28
	1.5 + 50 + 440	27 \pm 4	520 \pm 91	254 \pm 33
NaN_3 + H_2O_2 + α -ketoglutarate	1.5 + 340 + 500	cyt.	cyt.	321 \pm 73
	1.5 + 50 + 500	37 \pm 10	601 \pm 51	268 \pm 60
	1.5 + 340 + 600	cyt.	cyt.	266 \pm 71
	1.5 + 50 + 600	22 \pm 4	530 \pm 53	247 \pm 31

* cyt = cytotoxic

¹ The results are means of 2 separate experiments with 6 plates each.

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