Acta Biochimica Polonica

Vol. 42 No. 3/1995

291–296

QUARTERLY

Studies on the mechanism of mutagenicity and genotoxicity induced by dihydralazine

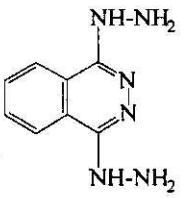
Bożena Chłopkiewicz, Anna Ejchart and Jadwiga Marczewska

Drug Institute, Chełmska 30/34, 00–725 Warsaw, Poland

Received: 22 December, 1994; accepted: 10 June, 1995

Key words: dihydralazine, mutagenicity, genotoxicity, active oxygen species

Dihydralazine was found to be mutagenic towards *S. typhimurium* TA1537, TA97, TA1538 and TA98 and genotoxic towards *E. coli* PQ37. Using the nitro blue tetrazolium reduction method we have found that dihydralazine can generate active oxygen species. The possible role of active oxygen species in mutagenicity (Ames test) and genotoxicity (SOS Chromotest) of dihydralazine was studied by testing the influence of the different active oxygen species scavengers on these two processes. Of the active oxygen scavengers tested, only superoxide dismutase suppressed partially the mutagenic and genotoxic activity of dihydralazine. This result seems to indicate that superoxide anion play a role in these two biological events.



Dihydralazine, a hydrazinophthalazine derivative, was discovered many years ago and is still extensively used in the treatment of chronic hypertensive disease. Some experimental data have indicated that dihydralazine possesses mutagenic and genotoxic activities. The drug was positive in the Ames reversion test [1, 2], in the HPC/DNA repair test [1], and induced a statistically significant increase in the frequency of sister chromatid exchanges [2]. It causes DNA fragmentation in mouse liver, lung, kidney and spleen [2]. The selective lethality assay in battery of five *E. coli* strains confirmed the direct genotoxicity of dihydralazine [2]. However, the molecular mechanism of biological effects induced by dihydralazine is not clear at that time.

The reactive oxygen species can play an important role in etiology of many diseases. They are formed during normal metabolic processes and also during exposure of organism to radiation or various chemicals (e.g. drugs). Reactive oxygen species have been implicated in mutagenesis and carcinogenesis [3].

It is known that hydrazine derivatives can act as free radical activators [4, 5]. Some authors postulate that dihydralazine activates human granulocytes *via* stimulation of hydroxyl radical production [6]. In our research we have attempted to determine whether active oxygen species generated by dihydralazine participate in its mutagenic and genotoxic effects.

MATERIALS AND METHODS

Chemicals. Dihydralazine (Nepresol) was from Ciba. Catalase (EC 1.11.1.6, from bovine liver), superoxide dismutase (EC 1.15.1.1, from bovine erythrocytes), *o*-nitrophenyl-β-D-galactopyranoside (the substrate for β-galactosidase), *p*-nitrophenylphosphate (the substrate for alkaline phosphatase) and nitro blue tetrazolium (NBT) were obtained from Sigma. Thiourea and D-mannitol were from Aldrich. All substances tested were dissolved in water.

Abbreviations used: HPC, hepatocyte primary culture; NBT, nitro blue tetrazolium.

Mutagenicity assay. *Salmonella typhimurium* tester strains used were TA1537, TA97, TA1538, TA98, TA1535, TA100, TA102 and TA104. The plate incorporation assay was carried out as described by Maron & Ames [7]. Scavengers of reactive oxygen species were added to the top agar together with dihydralazine.

Genotoxicity assay. The SOS Chromotest with *Escherichia coli* PQ37 was performed according to Quillardet & Hofnung [8]. Scavengers of reactive oxygen species were added to the tester strain together with dihydralazine. The substance was recognized as genotoxic when the β-galactosidase Induction Factor was greater than 1.5.

Measurement of active oxygen species formation. Formation of active oxygen species was measured by the reduction of NBT to formazan according to Beauchamp & Fridovich [9]. The reaction mixture containing 30 μM NBT and dihydralazine in 15 mM carbonate buffer (pH 10.2) was incubated in the presence or absence of various concentrations of scavengers of active oxygen species, and the rate of formazan formation was assayed by the rise in absorbance at 560 nm.

RESULTS AND DISCUSSION

The results concerning mutagenic activity of dihydralazine are summarized in Table 1. The Ames plate test assay showed a positive response to dihydralazine in strains TA1537, TA1538, TA97 and TA98, whose auxotrophy was caused by frameshift mutations. Of the remaining strains tested that became auxotrophic by substitution mutations, only TA102 was sensitive to high concentration (2000 μg/plate) of dihydralazine. These results differ from data presented by others. De Flora [2] and Glatt [10] showed that dihydralazine is mutagenic in base substitution strains TA100 and TA1535. Dihydralazine was not tested in strain TA102 till now. The positive results obtained with that strain, which is very sensitive to the action of peroxides and active oxygen [11] may suggest that the dihydralazine induced mutagenicity is connected with production of active oxygen species.

The possible contribution of active oxygen species in mutagenicity of dihydralazine was studied in *S. typhimurium* TA97 strain with different scavengers. Figure 1A shows the results of mutagenicity of dihydralazine in the presence of $HO^{\bullet}$ scavengers, D-mannitol and thiourea. The exposure of bacterial cells to dihydralazine produced revertants in a dose-dependent fashion. The addition of D-mannitol (30 mM) or thiourea (70 mM) to top agar together with dihydralazine did not reduce the mutagenic effect of dihydralazine. Similarly, addition of the H_2O_2 scavenger, catalase (2000

Table 1

Evaluation of dihydralazine mutagenicity with different strains of S. typhimurium (Ames test).

The mutagenicity assay of dihydralazine was carried out in triplicate without metabolic activation and the number of revertants was scored after incubation for 48 h at 37°C. The number of revertants per plate represents means for three experiments ± S.D.

Dihydralazine (μg/plate)	Number of revertants *his*$^+$/plate							
	TA1537	TA97	TA1538	TA98	TA1535	TA100	TA102	TA104
0	9 ± 3	146 ± 14	11 ± 3	25 ± 9	17 ± 5	170 ± 6	366 ± 55	421 ± 23
125	16 ± 4	182 ± 6	17 ± 2	37 ± 11	13 ± 2	183 ± 18	401 ± 23	496 ± 2
250	21 ± 6	248 ± 23	25 ± 5	49 ± 10	16 ± 4	163 ± 6	408 ± 81	442 ± 52
500	37 ± 5	312 ± 0	47 ± 8	72 ± 31	20 ± 3	184 ± 11	421 ± 13	395 ± 45
1000	81 ± 8	689 ± 72	42 ± 6	99 ± 11	20 ± 1	234 ± 19	512 ± 74	573 ± 83
2000	nt	1065 ± 140	nt	nt	0	61 ± 21	1164 ± 87	407 ± 28
3000	nt	nt	nt	nt	0	0	500 ± 47	0
4000	nt	nt	nt	nt	0	0	354 ± 32	0

nt, not tested

units) to the assay medium together with dihydralazine did not suppress dihydralazine induced mutagenicity (Fig. 1B). However, addition of the O_2^- scavenger, superoxide dismutase (300 units), gave a marked decrease in the number of dihydralazine induced revertants (Fig. 1C).

Dihydralazine was also found to be genotoxic as detected by SOS Chromotest (Table 2). Dihydralazine over the range of 125 μg to 1000 μg per sample induced β-galactosidase synthesis in *E. coli* PQ37 as a result of bacterial DNA damage. The SOS induction factor in treated cells was obtained from the rate of β-galactosidase and alkaline phosphatase activities compared to these rates in untreated cells. We investigated the influence of different active oxygen species scavengers on the SOS response induced in *E. coli* PQ37 by dihydralazine. The results presented in the same Table indicated

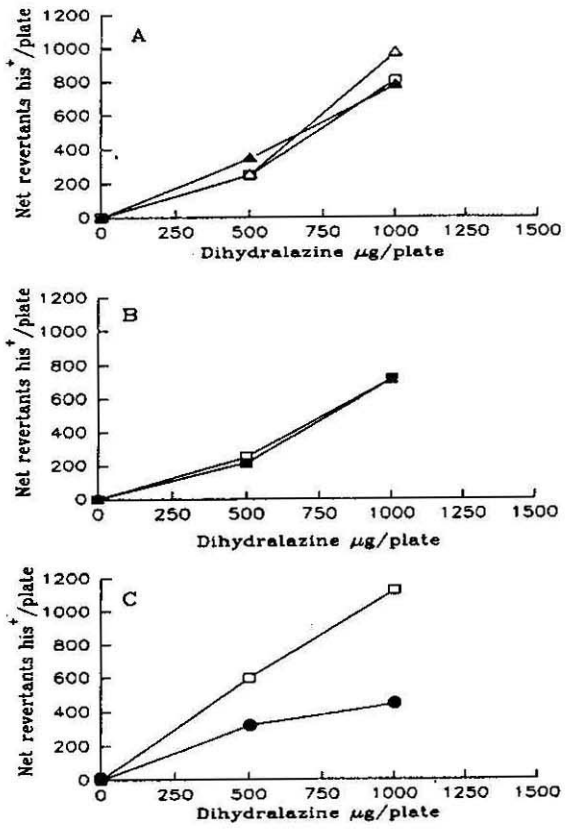


Fig. 1. Effects of some scavengers of active oxygen species on the mutagenicity of dihydralazine in Ames test with TA97.

Dihydralazine alone (□), dihydralazine with addition of D-mannitol 30 mM (Δ), thiourea 70 mM (▲), catalase 2000 units/plate (■), and superoxide dismutase 300 units/plate (●) were used.

that genotoxicity of dihydralazine was only weakly suppressed by the addition of D-mannitol, thiourea or catalase. In contrast superoxide dismutase reduced the activity of β-galactosidase induced by dihydralazine to about 50% of the control value.

The results presented above indicate that only O_2^- scavenger, superoxide dismutase, an enzyme that converts superoxide anion to less toxic oxygen metabolites, can affect mutagenic and genotoxic properties of dihydralazine. Therefore we postulate that superoxide anion plays a role in mutagenicity as well as in genotoxicity of dihydralazine. It is known that superoxide dismutase and catalase added to the assay medium cannot scavenge O_2^- and H_2O_2 generated inside the cells because these enzymes do not penetrate the cell membranes of bacteria. On the other hand, the cell wall is permeable to H_2O_2 and O_2^- (more to H_2O_2, less to O_2^-) [12]. Since superoxide dismutase added to the medium was able to protect partially bacterial cells against the genotoxic and mutagenic activities of dihydralazine, superoxide anion produced outside the cells seems to participate in mutagenicity and genotoxicity of the drug.

The possibility of generation of active oxygen species from dihydralazine outside the cells was tested by measuring reduction of NBT. Figure 2 shows that NBT was reduced by dihydralazine to formazan. The effect of active oxygen scavengers on the reduction rate of NBT by dihydralazine is shown in Fig. 3. The suppression of NBT reduction by the addition of scavengers might suggest that dihydralazine generates several active oxygen species. The experiments with NBT were carried out in basic pH (10.2) because NBT is not reduced in neutral pH. Also in neutral pH dihydralazine produces active oxygen species. It was confirmed in the experiment carried out in phosphate buffer, pH 7.4 with cytochrome *c*, which was reduced by dihydralazine (data not shown). However, only superoxide dismutase can effectively suppress the mutagenic and genotoxic activity of dihydralazine. These results indicate that the superoxide anion may play an important role in these biological events.

The question arises why the mutagenic and genotoxic effects of dihydralazine are only par-

Table 2

Genotoxicity of dihydralazine in the absence and presence of scavengers of active oxygen species (β-galactosidase Induction Factor in E. coli PQ37).

The values of Induction Factor represent means for three experiments ± S.D.

Scavenger	Induction Factor (% of inhibition) Dihydralazine (μg/sample)				
	0	125	250	500	1000
None		3.24 ± 0.97	4.06 ± 1.10	4.06 ± 1.50	3.72 ± 1.19
D-Mannitol 30 mM	0.91 ± 0.05	2.75 ± 0.13 (15)	3.67 ± 0.32 (10)	3.33 ± 0.21	3.31 ± 0.32 (11)
Thiourea 70 mM	1.08 ± 0.12	2.61 ± 0.34 (19)	3.07 ± 0.04 (23)	3.59 ± 0.16 (12)	3.09 ± 0.19 (17)
Catalase 889 U/sample	0.89 ± 0.09	2.62 ± 0.26 (19)	3.38 ± 0.16 (17)	3.96 ± 0.04 (2)	4.25 ± 0.13
Superoxide dismutase 300 U/sample	0.96 ± 0.11	1.62 ± 0.03 (50)	1.98 ± 0.04 (49)	2.19 ± 0.05 (54)	1.86 ± 0.12 (50)

The ratio of β-galactosidase units to alkaline phosphatase units after treatment with increasing concentrations of dihydralazine 125, 250, 500, 1000 μg/sample was 0.605, 0.758, 0.761, 0.666, respectively.

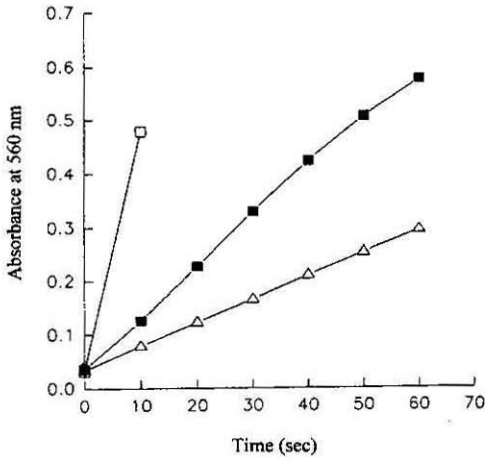


Fig. 2. Reduction of NBT by dihydralazine at concentrations of 100 μg/ml (□), 20 μg/ml (■) and 10 μg/ml (Δ).

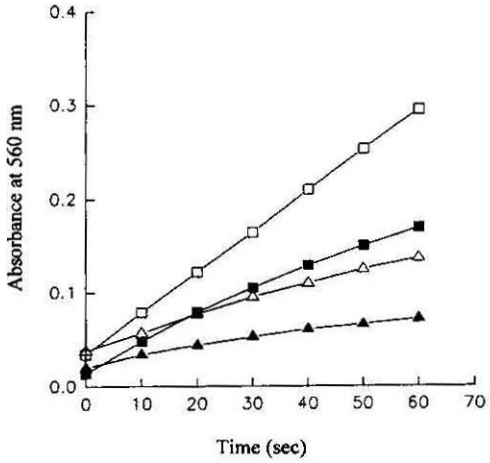


Fig. 3. Effect of some scavengers of active oxygen species on the reduction of NBT by dihydralazine. The reaction mixture contained 30 μM NBT with 10 μg/ml dihydralazine alone (□) or with addition of 500 units/ml superoxide dismutase (▲), 200 units/ml catalase (Δ) 35 mM thiourea (■).

tially affected by addition of superoxide dismutase. It is very likely that O_2^- formed intracellularly by dihydralazine is responsible for those effects but addition of dismutase to the medium can not influence intracellular processes. Since superoxide anion inside the cell can be scavenged by intracellular bacterial superoxide dismutase, it may be interesting to investigate the relationship between the level of intracellular superoxide dismutase and mutagenicity of dihydralazine. Oxidative damage in bacterial cells can occur due to the presence of a high level of superoxide anion generated by dihydralazine. On the other hand, another mechanism e.g. interaction of dihydralazine itself with DNA also might, in part, be responsible for its mutagenicity and genotoxicity. Such interaction has been demonstrated for another hydrazinophtalazine derivative, hydralazine [13].

REFERENCES

1. Williams, G.M., Mazue, G., McQueen, C.A. & Shimada, T. (1980) Genotoxicity of the antihypertensive drugs hydralazine and dihydralazine. *Science* **210**, 329–330.
2. De Flora, S., Zanacchi, P., Bennicelli, C., Camoirano, A., Cavanna, M., Sciaba, L., Cajelli,

E., Faggin, P. & Brambilla, G. (1982) *In vivo* and *in vitro* genotoxicity of three antihypertensive hydrazine derivatives (hydralazine, dihydralazine and endralazine). *Environ. Mutagenesis* **4**, 605–619.

3. Tudek, B. (1993) Oxydation of purines — the origin of spontaneous mutations. *Adv. Biochem.* **39**, 91–98.
4. Sinha, B.K. (1983) Enzymatic activation of hydrazine derivatives. *J. Biol. Chem.* **258**, 796–801.
5. Węglarz, L. & Bartosz, G. (1991) Hydralazine stimulates production of oxygen free radicals in Eagle's medium and cultured fibroblasts. *Free Radical Biol. Med.* **11**, 149–155.
6. Maliszewska, M., Jeziorski, A., Micera, G., Gąsiorowska, K. & Kozłowski, H. (1990) Influence of dihydralazine on the generation of hydroxyl radicals by human granulocytes. *Biomed. Biochim. Acta* **49**, 347–351.
7. Maron, D.M. & Ames, B.N. (1983) Revised methods for the *Salmonella* mutagenicity test. *Mutation Res.* **113**, 173–215.
8. Quillardet, P. & Hofnung, M. (1985) The SOS Chromotest, a colorimetric bacterial assay for genotoxins: procedures. *Mutation Res.* **147**, 65–78.
9. Beauchamp, C. & Fridovich, I. (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* **44**, 276–287.
10. Glatt, H. & Oesch, F. (1985) Propylazine is mutagenic in *Salmonella typhimurium* and *Escherichia coli*: distinct specificity for strains TA1537 and TA97. *Teratogenesis-Carcinogen-Mutagen.* **5**, 339–345.
11. Levin, D.K., Hollstein, M., Christman, M.F., Schwiers, E.A. & Ames, B.N. (1982) A new *Salmonella* tester strain (TA102) with AT base pairs at site of mutation detects oxidative mutagens. *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7445–7449.
12. Takahashi, M. & Asada, K. (1983) Superoxide anion permeability of phospholipid membranes and chloroplast thylakoids. *Arch. Biochem. Biophys.* **226**, 558–566.
13. Sinha, B.K. & Patterson, M.A. (1983) Free radical metabolism of hydralazine. Binding and degradation of nucleic acids. *Biochem. Pharmacol.* **32**, 3279–3284.