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THE HEMOLYTIC ACTIVITY OF CITRAL—II

GLUTATHIONE DEPLETION IN CITRAL TREATED ERYTHROCYTES

RUTH SEGAL* and ILANA MILO-GOLDZWEIG
Department of Natural Products, School of Pharmacy, Hebrew University, Jerusalem, Israel

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Abstract—The role of glutathione in citral induced hemolysis was investigated. The concentration of intracellular glutathione diminished in citral treated erythrocytes. GSH disappeared before hemolysis started. Glucose inhibited citral induced hemolysis and restored the depleted cellular stores of GSH. The results presented confirm the previous suggestion that a free radical-peroxide mechanism is involved in citral induced hemolysis.

It was recently reported [1–4] that the monoterpene citral has teratogenic effects on chick embryos. Morphological evidence indicated altered membrane functions which are possibly evoked by a lipid peroxidative mechanism [4]. We have suggested [5] that the hemolysis caused by citral in washed rat erythrocytes is also to be ascribed, at least in part, to lipid peroxidation of the polyunsaturated fatty acids of the cell membrane. Because of the extensive use of citral in the food and cosmetic industries [6, 7], further studies on the mechanism of its noxious effects are of interest. It was the aim of the present study to investigate the role of glutathione in citral induced hemolysis.

EXPERIMENTAL

Materials. All compounds were pure grade chemicals. Citral (*cis* and *trans*), was from Fluka A.G. Switzerland, Glutathione (reduced) from Mereck, Darmstadt, 5,5′-dithobis (2-nitorbenzoic acid) from Sigma Chemical Co., St. Louis, MO. Hemolysis tests were performed as described previously [8].

Reduced glutathione (GSH) determination. A modification of the method described by Beutler *et al.* [9] was applied. 3 ml of Na_2HPO_4 0.3 M and 0.5 ml of bisdithionitrobenzoate reagent [40 mg 5,5′-dithiobis(2-nitro benzoic acid) in 100 ml sodium citrate 1%] were added to 1 ml glutathione solution. The optical density was determined at 412 nm.

GSH determination in erythrocytes. The incubation mixture (100 ml) containing 1% erythrocytes was centrifuged at 4000 rpm for 10 min. The supernatant was removed. To the packed erythrocytes, 4.8 ml of metaphosphoric acid solution was added (1.7% metaphosphoric acid in 30% NaCl) under constant agitation. The suspension was left for 30 min at 0° for protein precipitation. Centrifuged at 4000 rpm for 10 min and the supernatant recentrifuged at 8000 rpm for 30 min at 4°. When the supernatant was not clear it was filtered through Whatman 50 filter paper. 1 ml of the supernatant was used for GSH determination.

RESULTS AND DISCUSSION

The level of reduced glutathione in erythrocytes which had been in contact with citral for various periods was determined. These experiments, which were conducted at high (5.9×10^{-4} M) and at low (3×10^{-4} M) citral concentrations, i.e. at high and at low rates of hemolysis, showed that in both cases GSH disappeared completely before hemolysis started. At high citral concentrations no GSH could be detected even after short periods of incubation (3–4 min) (results not presented). At low citral concentrations, however, a continuous reduction in GSH was observed until its complete disappearance (Fig. 1a). A plot of the progress of hemolysis under identical experimental conditions is presented in Fig. 1b. These two figures show that within the lag period, prior to citral hemolysis, a rapid decline in the glutathione level was obtained. Hemolysis started only some time after glutathione had disappeared completely.

Cellular stores of reduced glutathione can be depleted by two different mechanisms:

(a) A thioether conjugate can be formed with electrophilic agents in a reaction catalysed by

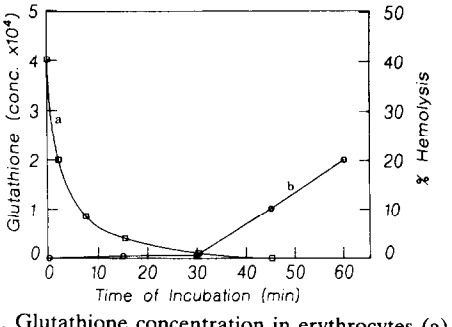

Fig. 1. Glutathione concentration in erythrocytes (a) and the extent of haemolysis (b) as function of incubation time with citral (3.5×10^{-4} M).

* To whom correspondence should be addressed.

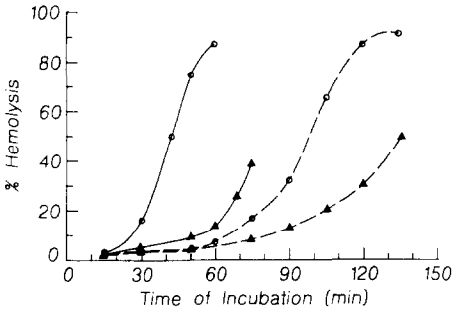

Fig. 2. Progress of citral induced hemolysis in the presence of glucose: O–O–O 4.7×10^{-4} M citral; ▲—▲—▲ 4.7×10^{-4} M citral with 2×10^{-1} M glucose; O – – O – – O 3×10^{-4} M citral; ▲ – – ▲ – – ▲ 3×10^{-4} M citral with 2×10^{-1} M glucose.

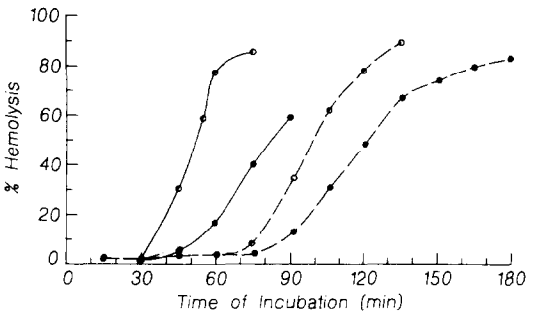

Fig. 3. The effect of low concentration of glutathione on the progress of citral induced hemolysis: O—O—O 4.5×10^{-4} M citral; ●—●—● 4.5×10^{-4} M citral with 3×10^{-4} M glutathione; O – – O – – O 3×10^{-4} M citral; ● – – ● – – ● 3×10^{-4} M with 3×10^{-4} M glutathione.

endogenous glutathione-*S*-transferase [10–12]. $\alpha\beta$-unsaturated carbonyl compounds, such as citral, are typical weak electrophiles that can produce this reaction. (b) The disappearance can also be due to the oxidation to glutathione disulphide. This oxidized GSSG is normally reduced by NADPH gained from glucose metabolism. When, however, excessive glutathione disulphide is produced, the normal glucose level in the erythrocyte may not suffice to produce all the NADPH needed for this reduction. In order to elucidate the mechanism of GSH disappearance we tested the effect which the addition of glucose to the incubation mixtures had on hemolysis and on the GSH level. The results (Fig. 2) show that glucose markedly inhibited the rate of hemolysis, both at high and at low citral concentrations, although it was more pronounced in the latter case.

Furthermore, the normal cellular GSH levels were regained in erythrocytes which after incubation with citral were washed and resuspended in buffer supplemented by glucose (1 mM). The quantity of the reduced GSH regained was a function of the length of time the erythrocytes were in contact with glucose (Table 1). No GSH could be detected in citral treated erythrocytes incubated with buffer alone. These results clearly show that GSH depletion was a result of its reversible oxidation. Inhibition of citral was also obtained by the addition of equimolar, and lower, concentrations of GSH (Fig. 3). Excesses of glutathione accelerated the hemolytic process (Fig. 4). This observation is in line with the suggestion that a peroxide mechanism is involved in citral hemolysis

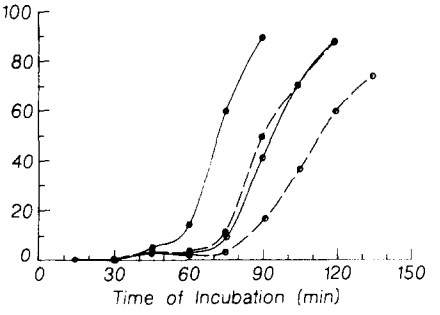

Fig. 4. The effect of high concentration of glutathione on the progress of citral induced hemolysis: O—O—O 3×10^{-4} M citral; ●—●—● 3×10^{-4} M citral with 5×10^{-3} M glutathione; O – – O – – O 2.4×10^{-4} citral; ● – – ● – – ● 2.4×10^{-4} citral with 5×10^{-3} M glutathione.

since GSH was previously shown [13] to trigger oxidative phospholipid destruction when incubated with isolated biomembranes under aerobic conditions.

The possibility that glucose and GSH inhibition of citral hemolysis were due to the transformation of citral to a reduced product could be excluded, since both inhibitors retarded, but did not abolish, the hemolytic effect. Thus, after exhaustion of the externally added reducing agents, citral started to exert its hemolytic effect.

Table 1. Concentration of reduced glutathione in erythrocytes after incubation with citral, centrifugation and reincubation in buffer-glucose (1 mM)

Citral concentration (M)	Time of incubation with citral (min)	Time of reincubation (min)	GSH concentration in erythrocytes (M) After reincubation with buffer	After reincubation with glucose (1×10^{-3} M)
–	–	–	–	4×10^{-4}
3×10^{-4}	7	15	–	4×10^{-4}
3×10^{-4}	7	15	–	4×10^{-4}
5×10^{-4}	30	3	–	1.6×10^{-4}
5×10^{-4}	30	5	–	3×10^{-4}
5×10^{-4}	30	10	–	4×10^{-4}

In our previous study on citral hemolysis [5] we suggested the coexistence of two distinct mechanisms: a free radical-peroxide induced mechanism which predominates at low citral concentration and a second mechanism predominating at high citral concentrations. This second type was ascribed to either an irreversible glutathione depletion which makes the red blood cells prone to oxidative challenge, or to a non-specific interaction of the monoterpene with the red blood cell membrane, similar to that of other terpenoids and steroids [14].

The results now presented confirm the existence of the free radical-peroxide mechanism. It furthermore indicates that no irreversible glutathione depletion is involved (Table 1). We may therefore assume a nonspecific interaction of the monoterpene with the erythrocyte membrane.

REFERENCES

1. A. Abramovici, in *Drugs and Fetal Development* (Eds. M. Klingberg, A. Abramovici and J. Chemke), p. 161. Plenum Press, New York (1972).
2. P. Rachmuth, E. Liban and A. Abramovici, *Teratology* **10**, 320 (1974).
3. A. Abramovici, J. Kam, E. Liban and R. Y. Barishuk, *Devl Neurosci* **1**, 177 (1978).
4. A. Abramovici, R. Rachmuth-Forschmidt, E. Liban and U. Sandbank, *J. Path.* **131**, 289 (1980).
5. I. Tamir, A. Abramovici, I. Milo-Goldzweig and R. Segal, *Biochem. Pharmac.* **33**, 2945 (1984).
6. P. Z. Bedoukian, in *Perfumery and Flavouring Synthetics*, 2nd edn., p. 99. Elsevier, Amsterdam (1967).
7. W. C. Menly, *Am. Perfum. Cosmet.* **85**, 123 (1970).
8. R. Segal, P. Shatkovsky and I. Milo-Goldzweig, *Biochem. Pharmac.* **23**, 973 (1974).
9. E. Beutler, O. Duron and B. M. Kelly, *J. Lab. clin. Med.* **61**, 882 (1963).
10. J. H. Harlan, J. D. Levine, K. S. Callahan, B. R. Schwartz and L. A. Harker, *J. clin. Invest.* **73**, 706 (1984).
11. C. J. Marcus, W. H. Habig and W. B. Jakoby, *Archs. Biochem. Biophys.* **188**, 287 (1978).
12. J. L. Plummer, B. R. Smith, H. Siesand and J. R. Bend, in *Methods in Enzymology*, Vol. 77 (Ed. B. Jakoby), pp. 50–59. Academic Press, New York (1981).
13. I. F. Koster and R. G. Slee, *Biochim. biophys. Acta* **752**, 233 (1983).
14. R. Segal, I. Milo-Goldzweig and M. Seiffe, *Life Sci.* **11**, II, 61 (1972).