Biochemical Pharmacology, Vol. 34, No. 8, pp. 1339–1341, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

An essential requirement for ferrous-haemoglobin in the hydrogen peroxide stimulated oxidation of red blood cell membrane lipids

(*Received* 13 *August* 1984; *accepted* 23 *October* 1984)

The red blood cell is a unique biological structure containing high concentrations of polyunsaturated fatty acids, molecular oxygen and ferrous ions in the ligand state. These components might be expected to make it highly susceptible to oxidative damage. In fact, several studies have shown that normal red blood cells are exceptionally resistant to oxidative changes [1]. Protection can be partly ascribed to cellular antioxidants, including catalase, superoxide dismutase and glutathione peroxidase; but it mainly reflects effective structural compartmentalisation of cellular constituents [2–4].

When red blood cells are incubated with high concentrations of hydrogen peroxide in the presence of a catalase inhibitor membrane lipids are slowly and partly oxidized [5]. The currently used hydrogen peroxide stress test based on the measurement of thiobarbituric acid (TBA) reactivity had provided a simple and sensitive method for clinical studies [6–12]. However, several aspects of the test and its interpretation remain unclear. In particular, the specificity of measurements based on TBA-reactivity has been questioned [13–14]. The mechanism of initiation of lipid peroxidation is further complicated by the finding that haemoglobin does not catalyse the superoxide dependent Fenton reaction (iron catalysed Haber-Weiss) [15] (eqn 1) and reacts with hydrogen peroxide. Iron salts, on the other hand, do produce hydroxyl radicals from hydrogen peroxide by a Fenton reaction (eqn 2).

$$O_2^{\cdot} + H_2O_2 \xrightarrow[\text{catalyst}]{\text{Fe}} OH^{\cdot} + OH^- + O_2 \qquad (1)$$

$$Fe^{2+} + H_2O_2 \longrightarrow OH^{\cdot} + OH^- + Fe^{3+} \qquad (2)$$

Hydroxyl radicals have the potential to initiate lipid oxidation by hydrogen abstraction although their contribution cannot be detected in the presence of an iron salt [16]. In the present study we have used a highly sensitive and specific method to measure lipid oxidation in red blood cells based on the evolution of hydrocarbon gases [17, 18]. The results strongly suggest that haemoglobin in an iron (II) state is an essential requirement before lipid oxidation can occur in the presence of hydrogen peroxide.

Materials and methods

Phenylhydroxylamine was purchased from Fluka (Buchs, Switzerland), all other chemicals from Merck (Darmstadt, F.R.G.). Microcristalline, α-cellulose, and haemoglobin were obtained from Sigma (Munich, F.R.G.). Blood was taken from normal healthy adults into sodium citrate as anticoagulant. Cells were separated from plasma by the method of Beutler *et al.* [19], with microcristalline and α-cellulose (1:1, w/w).

Experiments with red blood cells. Red cells were preincubated for 1 hr in phosphate buffered saline (PBS) pH 7.4 at 37° in a shaking water bath. The red cell suspensions were adjusted to haematocrit 2.5% by the addition of PBS containing 0.25 mM sodium azide. Red cell suspensions were treated with either sodium nitrate or phenylhydroxylamine for 15 min at 37° in a shaking water bath. After this procedure hydrocarbons were analyzed immediately in the head space of the incubation vials as described previously [18] and then red cells were washed three times with PBS pH 7.4 (phenylhydroxylamine experiments) or pH 8.0 (sodium nitrite experiments), in order to remove the methaemoglobin forming chemicals. Thereafter, methaemoglobin formation was estimated as described by Henry *et al.* [20]. Susceptibility of red cells to lipid peroxidation was monitored by the release of pentane and ethane into the head space of 10 ml-head-space-vials in which 3 ml red cells (haematocrit 2.5%) were treated at 37° for 2 hr with PBS pH 7.4 containing 10 mM hydrogen peroxide and 0.25 mM azide. Analysis of hydrocarbons in the head space was performed by gas chromatography as previously described [17, 21]. After 2 hr of red cell incubation with hydrogen peroxide, haemolysis was determined by measuring the haemoglobin concentration in the supernatant of red cell suspensions photometrically after conversion to cyanmethaemoglobin at 540 nm.

Experiments with red cell membranes and haemoglobin. Red cell ghost membranes were isolated as described by Burton *et al.* [23]. Haemoglobin (Sigma, human type IV) contains 97% methaemoglobin. In experiments with oxyhaemoglobin dithionite (0.4 g/l haemoglobin suspension) was added and pure oxygen was bubbled through the haemoglobin for 5 min. After the latter procedure haemoglobin was found to be 99% in the iron (II) state. Ghosts and met- or oxyhaemoglobin were suspended in PBS pH 7.4 and adjusted to a concentration of 0.1 mg membrane protein (estimated as described by Lowry *et al.* [22]) per ml and 0.8 mg haemoglobin per ml suspension. Three millilitres of the ghost and haemoglobin containing suspension were introduced into the head space vials and incubated for 2 hr at 37° in a shaking water bath after initiating lipid peroxidation by the addition of 10 mM hydrogen peroxide (final concentration). Hydrocarbons were analysed as described previously [17, 21].

Results and discussion

Oxidation of 30–40% of oxyhaemoglobin to methaemoglobin was achieved by incubation of red cells with either 2 mmole/l sodium nitrite or 400 μmole/l phenylhydroxylamine for 15 min. After incubation of red cells with either phenylhydroxylamine (25–400 μmole/l) or nitrite (0.5–2.0 mmole/l) hydrocarbons were not detectable in the head space of the incubation vials.

The haemolysis occurring after the formation of various methaemoglobin concentrations and subsequent incubations of red cells with 10 mmol/l hydrogen peroxide in the presence of 0.25 mmol/l azide for 2 hr was shown in Table 1. Haemolysis was not detectable in experiments performed with red cells containing more than 40% methaemoglobin prior to the hydrogen peroxide exposure.

Amounts of pentane formed during breakdown of red cell membrane fatty acids due to oxidant stress were illustrated in Figs. 1 and 2. Increasing methaemoglobin content of red cells led to decreasing pentane generation. Like haemolysis, lipid peroxidation was not detectable in experiments performed with red cells containing approximately 40% methaemoglobin.

In order to exclude that these results were due to the irreversible oxidation of molecules other than haemoglobin by nitrite or phenylhydroxylamine, we carried out experiments with red cell ghost membranes and haemoglobin alone. Methaemoglobin content of the haemoglobin used (Sigma, human type IV) amounted to 97%. Incubation of this haemoglobin and ghost membranes in the presence of

Table 1. Haemolysis of red cells after hydrogen peroxide-induced lipid peroxidation

Pretreatment of red cells	Haemolysis (%)	N
Controls	35.8 ± 0.7	38
Sodium nitrite (mM)		
0.5	25.4 ± 6.3	6
1.0	14.7 ± 4.5	6
1.5	5.4 ± 2.1	6
2.0	0	6
Phenylhydroxylamine (μM)		
25	29.6 ± 8.1	3
50	21.1 ± 5.8	5
100	18.4 ± 6.6	6
200	6.6 ± 5.8	6
400	0	6

Pretreatment of red cells was performed for 15 min at 37°. Haemolysis was checked in the supernatant of red cell suspensions after an incubation with 10 mM hydrogen peroxide in the presence of 0.25 mM azide in PBS pH 7.4 for 2 hr at 37°. Mean values ± S.D. N = number of experiments with red cells from different donors.

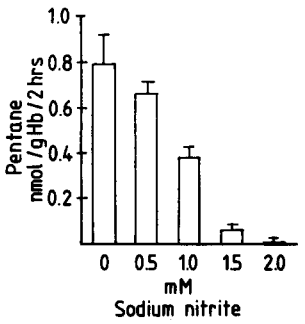

Fig. 1. Pentane formation of red cells incubated for 2 hr with 10 mM hydrogen peroxide in the presence of azide (0.25 mM). Pretreatment of red cells with various concentration of sodium nitrite. Mean values ± S.D. Numbers of experiments indicated in Table 1.

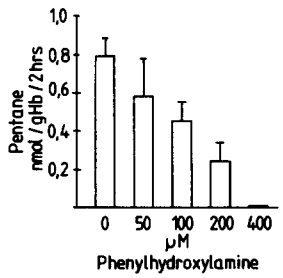

Fig. 2. Pentane formation of red cells incubated for 2 hr with 10 mM hydrogen peroxide in the presence of azide (0.25 mM). Pretreatment of red cells with various concentration of phenylhydroxylamine. Mean values ± S.D. Numbers of experiments indicated in Table 1.

10 mmole/l hydrogen peroxide did not yield a detectable amount of pentane. By contrast, in experiments with oxyhaemoglobin obtained by the reduction of methaemoglobin with dithionite and dioxygen, pentane was formed (Table 2).

Table 2. Lipid peroxidation of red cell ghost membranes by hydrogen peroxide

Incubation	Pentane
Ghosts (0.1 mg protein/ml) + H_2O_2 (10 mM)	0
Haemoglobin (0.8 mg/ml) + H_2O_2 (10 mM)	0
Ghosts (0.1 mg protein/ml) + Haemoglobin (0.8 mg/ml) + H_2O_2 (10 mM)	0
Ghosts (0.1 mg protein/ml) + Dithionite (0.4 mg/ml)	0
Haemoglobin (0.8 mg/ml) + Dithionite (0.4 mg/ml) + O_2	0
Ghosts (0.1 mg protein/ml) + Haemoglobin (0.8 mg/ml) + Dithionite (0.4 mg/ml) + O_2 + H_2O_2 (10 mM)	0.8

Suspensions (PBS, pH 7.4) were performed for 2 hr at 37° in a shaking water bath. Three millilitres of incubation medium were introduced in a 10-ml head space vial. In experiments with O_2, it was bubbled through the incubation medium for 5 min. Mean of 3 experiments. Pentane expressed as nmole/mg membrane protein.

These results suggest that haem iron is an effective catalyst for red cell membrane lipid peroxidation only in its reduced ligand state in experiments performed with hydrogen peroxide. In other words the membrane lipids of methaemoglobin-containing red cells are relatively protected against peroxidation. Haem-II-iron seems to be an essential requirement for red cell lipid peroxidation induced by hydrogen peroxide. Further investigations should clarify if this requirement may apply to other oxidative mechanisms, e.g. by phenylhydrazine.

Eberhard-Karls-Universität Tübingen
Medizinische Klinik, Abt. II and Institut für Toxikologie,
Federal Republic of Germany

MICHAEL R. CLEMENS
HERMANN EINSELE
HERBERT REMMER†
HANS DIERCK WALLER

REFERENCES

1. T. L. Dormandy, *Ann. R. College Surg.* **62**, 188 (1980).
2. T. L. Dormandy, *Lancet* **i**, 647 (1978).
3. J. S. Bus and J. E. Gibson, in *Lipid Peroxidation and its Role in Toxicology* (Ed. E. Hodgson, J. R. Bend and R. M. Philpot), *Rev. Biochem. Toxicol*, p. 125, Elsevier, New York (1979).
4. P. Hochstein, Antioxidant mechanisms associated with lipid peroxidation, Proc. IIIrd Intern. Conf. Hyperbaric Oxygen, publication 1404, p. 61, Nat Acad Sci, Washington D.C. (1966).
5. J. Stocks and T. L. Dormandy, *Br. J. Haemat.* **20**, 95 (1971).
6. J. Stocks, M. Kemp and T. L. Dormandy, *Lancet* **i**, 266 (1971).
7. S. K. Das and R. C. Nair, *Br. J. Haemat.* **44**, 87 (1980).
8. M. I. S. Hunter, M. S. Brzeki and P. J. de Vane, *Clin. Chim. Acta* **115**, 93 (1981).
9. J. Stocks, E. L. Offerman, C. B. Modell and T. L. Dormandy, *Br. J. Haemat.* **23**, 713 (1972).

10. B. Matkivics, A. Laszlo and L. Szabo, *Clin. Chim. Acta* **118**, 289 (1982).
11. D. P. Chanhan, P. H. Gupta, M. R. N. Nampoothiri, P. C. Singhal, K. S. Chugh and C. R. Nair, *Clin. Chim. Acta* **123**, 153 (1982).
12. T. P. Flynn, D. W. Allen, G. J. Johnson and J. P. White, *J. clin. Invest.* **71**, 1215 (1983).
13. J. M. C. Gutteridge, *FEBS Lett.* **128**, 343 (1981).
14. J. Stocks and T. L. Dormandy, *Clin. Chim. Acta* **27**, 117 (1970).
15. B. Halliwell, *FEBS Lett.* **92**, 321 (1978).
16. J. M. C. Gutteridge, *FEBS Lett.* **150**, 454 (1982).
17. M. R. Clemens and H. Remmer, *Blut* **45**, 329 (1982).
18. M. R. Clemens, H. Einsele, H. Frank, H. Remmer and H. D. Waller, *Biochem. Pharmac.* **32**, 3877 (1983).
19. E. Beutler, C. West and K. G. Blume, *J. lab. Clin. Med.* **88**, 328 (1976).
20. R. Henry, D. C. Cannon and J. W. Winkleman, in *Clinical Chemistry, Principles and Practice*, p. 1149. Harper and Row, New York (1974).
21. H. Frank, T. Hintze, D. Bimboes and H. Remmer, *Toxic. appl. Pharmac.* **56**, 337 (1980).
22. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
23. G. W. Burton, K. U. Ingold and K. E. Thompson, *Lipids* **16**, 946 (1981).

Biochemical Pharmacology, Vol. 34, No. 8, pp. 1341–1343, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

Thyroliberin (TRH), 6-methyl-5-oxothiomorpholinyl-3-carbonyl histidylproline amide and histidylproline diketopiperazine do not affect the release of [^{3}H]-acetyl choline and [^{3}H]-choline from rat brain tissue cubes

(*Received* 25 *June* 1984; *accepted* 13 *November* 1984)

Thyroliberin (L-pyroglutamyl-L-histidyl-L-proline amide, TRH) elicits a variety of behavioural changes [1] in addition to its role as a hypophysiotrophic hormone. The effect of TRH on locomotor activity, mediated by the mesolimbic dopaminergic pathway [2, 3] and its antagonism of drug-induced narcosis [4, 5] possibly mediated by the septal–hippocampal cholinergic pathway [6] have been particularly well studied.

Several studies have sought a biochemical basis for some of the TRH-induced behavioural changes. One of the most frequently reported results is that 10^{-5}–10^{-3} M TRH stimulates the release of dopamine from nucleus accumbens [3, 7] and striatal [8, 9] preparations. In view of the relatively high concentrations required, it is unlikely that TRH has a direct effect on dopamine release. TRH has been reported to stimulate the release of acetylcholine (ACh) from rabbit cerebral cortex [6]. It was decided, therefore, to test an alternative hypothesis that the prime effect of TRH is to stimulate ACh release, which in turn enhances DA release from the nucleus accumbens [10] and striatum [11]. In particular an effect of TRH on the release of ACh from the septum might provide a biochemical basis for the TRH-induced antagonism of pentobarbital and ethanol narcosis [6].

Few reports have appeared on the effect of TRH on cholinergic biochemistry. This study finds that TRH, its analogue 6-methyl-5-oxothiomorpholinyl-3-carbonylhistidyl proline amide (CG3703) and a metabolite histidylproline diketopiperazine (cyclo His-Pro) do not effect the release of either ACh or Ch from rat brain nucleus accumbens or septal tissue cubes.

Materials and methods

Male Wistar rats (250 ± 50 g) were decapitated, their brains rapidly removed and nuclei accumbens dissected according to the method of Horn *et al.* [12]. Septa were dissected by making a coronal section at the optic chiasma and a second section 3 mm caudal to the optic chiasma. The slice of tissue was placed rostral face uppermost and the septal region dissected as a cube (90–120 mg wet weight), with 2–3 mm^2 cross section, on the mid line, immediately ventral to the corpus callosum, and between the corpora striata.

Choline uptake and release. Tissue chops (0.2 mm × 0.2 mm) were obtained from each brain region, using a McIlwain tissue chopper, and incubated in Tris-buffered saline (50 mg tissue/ml) consisting of 20 mM Tris–HCl, pH 7.4 containing 120 mM NaCl; 5 mM KCl; 3.5 mM $NaHCO_3$; 2.5 mM $CaCl_2$; 1.2 mM KH_2PO_4; 1 mM $MgSO_4$; 20 mM glucose; 0.5 mM asorbic acid; 0.1 mM physostigmine; 50 μg/ml bacitracin and 0.17 μM [^{3}H]-choline chloride (10 μCi; sp. act 60 Ci/mmole) at 37° with shaking for 30 min. Uptake was terminated by centrifugation at 10,000 *g* for 10 min at 4°, the pellet washed three times in 5 ml Tris-buffered saline and resuspended in the same medium (600 μl) per nucleus accumbens or septum). Aliquots (600 μl) were transferred to Eppendorf microcentrifuge tubes and the brain fragments pelleted by centrifugation at 10,000 g for 2 min at 20°. The tubes were placed on ice and the supernatant removed by aspiration. Tris-buffered saline (600 μl) at 37°, containing either 5 mM KCl or 50 mM KCl, in the presence or absence of 0.1 mM TRH, 0.1 mM cyclo His Pro or 0.1 mM CG 3703, was added to each tube, which were then incubated, with frequent mixing, at 37° for 5 min. Release of [^{3}H]-compounds was terminated by cooling the tubes to 0° followed by centrifugation at 10,000 *g* for 2 min. Aliquots (400 μl) of each supernatant were lyophilized, the residue dissolved in 50 μl methanol containing 10 mM choline (Ch) and 10 mM acetylcholine (ACh) as standards, and the proportion of radioactivity in Ch and ACh determined by chromatography of 10 μl samples on cellulose TLC plates. The total radioactivity released was determined by removing 100 μl aliquots of the supernatant for liquid scintillation counting using toluene–Triton X-100 (2:1 v/v) containing 0.8% (w/v) PPO as scintillant. The remainder of the supernatant was removed, by aspiration, and the pellets were homogenized in methanol (150 μl) containing 10 mM Ch and 10 mM ACh, using a mini-drill with a Teflon-tipped pestle. The homogenate was stored at −20° for 30 min and the protein precipitate removed by centrifugation at 10,000 *g* for 2 min. The total radioactivity, extracted from the pellet, was measured by removing aliquots (100 μl) of the supernatant for liquid scintillation spectrometry. The proportion of tritium in Ch and ACh determined by chromatography of 10 μl aliquots of the supernatant on cellulose TLC plates.

Release of [^{3}H]Ch and [^{3}H]ACh was expressed as:

$$\frac{\text{cpm in supernatant}}{\text{cpm in Supernatant + cpm in pellet}} \times 100\%$$

Statistical significance was determined using the Student's *t*-test [13].