Biochemical Pharmacology, Vol. 33, No. 11, pp. 1715–1718, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

PHENYLHYDRAZINE-INDUCED LIPID PEROXIDATION OF RED BLOOD CELLS *IN VITRO* AND *IN VIVO*: MONITORING BY THE PRODUCTION OF VOLATILE HYDROCARBONS

MICHAEL R. CLEMENS,* HERBERT REMMER† and HANS DIERCK WALLER*

*Medizinische Klinik, Abt. Innere Medizin II and †Institut für Toxikologie, Eberhard-Karls-Universität Tübingen, D-7400, Federal Republic of Germany

(*Received* 27 *July* 1983; *accepted* 16 *November* 1983)

Abstract—Human red blood cells and male Sprague–Dawley rats were treated *in vitro* and *in vivo*, respectively, with phenylhydrazine in order to determine whether the release of volatile hydrocarbons can serve as a suitable index for phenylhydrazine-induced red blood cell peroxidation. Lipid peroxidation following phenylhydrazine administration (*in vitro* experiments: dosage calculated at 0.5–50 mM; *in vivo* experiments: intraperitoneal injection of 2.8 mg/100 g body wt) was monitored by the release of ethane and pentane measured by gas chromatography. Further hydrocarbons such as ethylene, propane, *n*-butane, *iso*-butane and *iso*-butene were monitored to form a basis of comparison. *In vitro* haemolysis was also determined during the course of incubation. Red blood cell suspensions yielded more than 15-fold concentrations of propane and more than 2-fold concentrations of *iso*-butane compared to pentane and ethane yields. Haemoglobin solutions also produced propane and *iso*-butane in the presence of phenylhydrazine, whereas pentane and ethane were not detectable. Time-course studies revealed that ethane and pentane reached maximum *in vitro* levels after red blood cell suspensions had been incubated for 2 hr whereas the maximum degree of haemolysis (approximately 60%) was attained between 60 and 90 min following the beginning of phenylhydrazine treatment. The dosage did not affect the final degree of haemolysis. Rats treated with phenylhydrazine exhaled greater concentrations of ethane (6-fold increase) and pentane (2-fold increase) compared to control animals. Exhaled propane showed a 30-fold increase in concentration following drug treatment. Our results suggest that the release of pentane and ethane may be useful in assessing red blood cell lipid peroxidation in the presence of phenylhydrazine *in vitro* and *in vivo*.

Phenylhydrazine, long known as a haemolytic agent [1], is a valuable tool in experimental model systems which study the mechanisms producing chemically induced cell damage, and evaluate the biological defence mechanisms of red blood cells counteracting free radical-mediated cell damage. The haemolytic effect of phenylhydrazine has been attributed to its autoxidation, the subsequent oxidation of enzymes, membrane proteins and haemoglobin, and its initiation of peroxidation reactions in membrane phospholipids [2]. Some investigators [3–5] have observed evidence that superoxide radicals may be involved in the haemolytic action of phenylhydrazine. Cohen and Hochstein [6] have shown that phenylhydrazine generates hydrogen peroxide in red blood cells.

Lipid peroxidation of red blood cells is currently studied *in vitro* by measuring the formation of malondialdehyde and fluorescent chromolipids. The release of short-chain volatile hydrocarbons from fatty acid hydroperoxides has recently been proposed as a reliable, sensitive index for lipid peroxidation *in vivo* and *in vitro* [7–14]. We have recently observed the release of volatile hydrocarbons formed during *in vitro* lipid peroxidation resulting from hydrogen peroxide-induced red blood cell peroxidation [15].

Hence, we examined the question whether the release of volatile hydrocarbons can serve as a suitable index for phenylhydrazine-induced red blood cell lipid peroxidation *in vitro* and *in vivo*.

MATERIALS AND METHODS

All chemicals used were of analytical grade. Haemoglobin (human type IV, containing *ca.* 75% methaemoglobin) and celluloses were purchased from Sigma (Munich, F.R.G.); phenylhydrazine from EGA (Aldrich Co., Steinheim, F.R.G.).

Experiments in vitro. Blood was drawn from healthy donors using 3.13% sodium citrate as anticoagulant (1:5, v/v). Cells were isolated using isoosmotic phosphate-buffered saline (PBS, pH 7.4), microcrystalline cellulose and α-cellulose (1:1, w/w) as described by Beutler *et al.* [16]. Red blood cells were suspended in PBS and preincubated in glass test-tubes in a shaking water bath for 1 hr at 37°, after which haematocrit was adjusted to 2.5% by adding PBS. Samples containing haemoglobin were adjusted to 8 mg/ml haemoglobin in PBS, i.e. a concentration comparable to that used in experiments with red cells.

Varying amounts of phenylhydrazine, ranging from 0.5 to 50 mM as indicated in the legends were added to the red blood cell or haemoglobin samples. Then 3 ml of each sample was aliquoted into 10 ml headspace vials, and the vials were immediately sealed with special air-tight rubber septums. Headspaces were flushed with hydrocarbon-free synthetic air for 2 min in order to replace laboratory air containing small amounts of hydrocarbons. The sealed

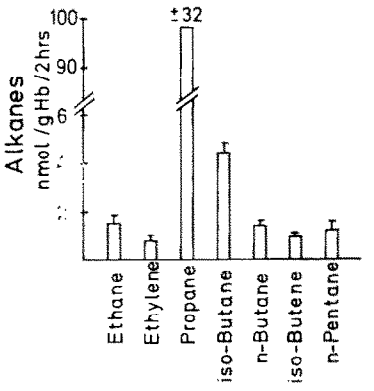

Fig. 1. Production of hydrocarbons upon incubation of red cells (haematocrit 2.5%) with 5 mM phenylhydrazine for 2 hr at 37°. Mean values of six experiments ± S.D. Performed with red cells drawn from different donors.

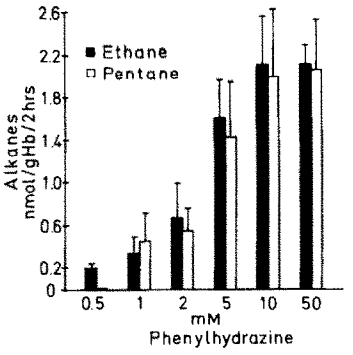

Fig. 2. Dose–response relationship of hydrocarbon production to various concentrations of phenylhydrazine. Incubation of red cell suspensions (haematocrit 2.5%) for 2 hr at 37°. Mean values of six experiments ± S.D. Performed with red cells drawn from different donors.

samples were then incubated in a shaking water bath at 37°. After certain periods, 1 ml of the gas phase was drawn with a gas-tight syringe (Unimetrics) and injected into the gas chromatograph, as described previously [17]. The degree of haemiolysis was determined by measuring the haemoglobin concentration photometrically in the supernatant of the red cell suspensions after conversion to cyanmethaemoglobin at 540 nm.

Experiments in vivo. Male Sprague–Dawley rats (Ivanovas, Kisslegg, F.R.G.), weighing 200–220 g were fed a standard diet of Altromim (Lage, F.R.G.) and provided with tap water *ad lib.* They were maintained in air-conditioned rooms with controlled 12 hr dark–light cycles and were fasted overnight before the experiments. Each rat was injected either with 0.5 ml saline and used as a control, or with 0.5 ml saline containing 2.8 mg phenylhydrazine per 100 g body wt. The animals were then immediately placed in a closed desiccator system for the analysis of exhaled hydrocarbons, as described previously [17, 18]. Gas samples (10 ml) were drawn from the desiccator system and injected into the gas chromatograph immediately and after 1 hr periods until the 4th hr of drug treatment.

RESULTS

Effects of phenylhydrazine in vitro

The addition of phenylhydrazine to red blood cells produced volatile hydrocarbons (Fig. 1). Unlike our previous experiments with hydrogen peroxide, large quantities of propane were detected. *Iso*-butane levels were approximately twice as high as those of either ethane or pentane. Haemoglobin, on the other hand, yielded no detectable amounts of ethane or pentane in the presence of phenylhydrazine, while propane and *iso*-butane levels were approximately equal to those of red blood cell suspensions (Table 1).

Table 1. Production of alkanes from haemoglobin

Pentane	Ethane	Propane	iso-Butane
		nmol/g Hb/2 hr	
0	0	91.3 ± 0.9	8.9 ± 0.1

Incubation of haemoglobin (0.8 g/dl) with 2 mM phenylhydrazine. Mean values ± S.D. of three experiments.

Figure 2 illustrates the dose–response relationship in red blood cell solutions with regard to the production of ethane and pentane. Maximum response was attained with a dose of 50 mM phenylhydrazine. The ratio of pentane to ethane was approximately 1.

Figure 3 illustrates the time courses of ethane and pentane released following 5 and 10 mM doses of

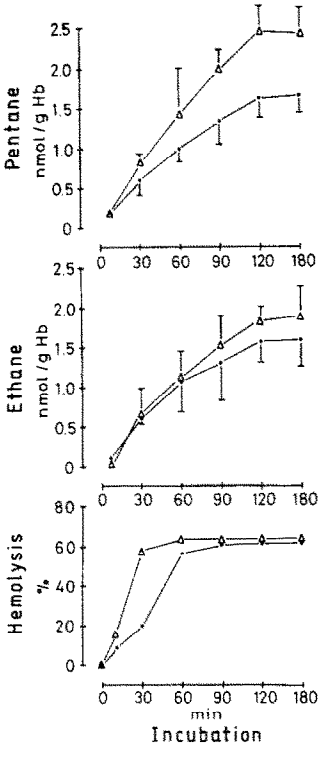

Fig. 3. Hydrocarbon production and degree of haemolysis during incubations of red cells (haematocrit 2.5%) with 5 (●) and 10 (▲) mM phenylhydrazine. Mean values of five experiments ± S.D. Performed with red cells from different donors.

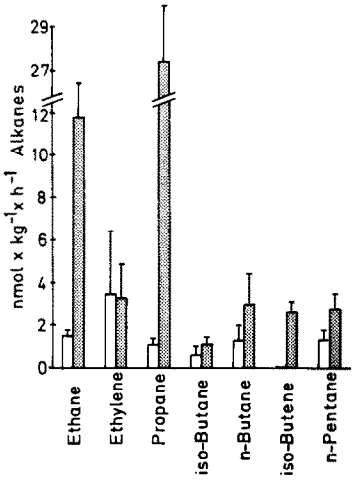

Fig 4. Exhalation of hydrocarbons per hr (mean of 3 hr) of control animals (white areas) and phenylhydrazine-treated animals (2.8 mg/100 g body wt) (pointed areas). Mean values of eight animals in each group ± S.D.

phenylhydrazine, as well as the degree of haemolysis in red blood cell suspensions measured throughout the course of incubation. Maximum haemolysis amounted to approximately 60%, it occurred after 60 min incubation, and did not increase regardless of the chosen drug concentration. Concentrations of ethane and pentane reached a maximal level after an incubation period of 120 min. Time courses of the remaining hydrocarbons were comparable to those obtained for ethane and pentane (data not shown); concentrations after 2 hr of incubation are illustrated in Fig. 1.

Effects of phenylhydrazine in vivo

Figure 4 summarizes the exhalation of hydrocarbons by male Sprague–Dawley rats. The propane levels obtained were found to be formed *in vivo* as efficiently as those obtained under *in vitro* conditions. Phenylhydrazine-treated animals exhaled greater quantities of ethane (6-fold increase) and pentane (2-fold increase) than control animals. Ethylene levels exhaled by the control animals and drug-treated animals were the same. The control animals did not exhale detectable quantities of *iso*-butene, whereas treated animals exhaled *iso*-butene in quantities comparable to those of pentane and *n*-butane.

DISCUSSION

The peroxidation of polyunsaturated fatty acids present in the membrane lipids is considered to be a basic mechanism of toxicity for a wide variety of chemicals, but its role in red cell haemolysis remains obscure. The biochemical consequences of lipid peroxidation are well known and include membrane damage, inhibition of enzyme function, and cross-linking of proteins [19]. The relationship of lipid peroxidation and haemolysis *in vitro* and in particular *in vivo* is difficult to evaluate due to a lack of suitable methods.

Lipid peroxidation in biological tissues has been investigated by measurement of the major peroxidation products such as hydroperoxides, conjugated dienes, malondialdehyde, as well as hexanal and fluorescent carbonylamine products. A suitable technique to measure lipid peroxidation *in vivo* has been introduced with the analysis of volatile hydrocarbons, such as pentane and ethane, which are formed upon the decomposition of unsaturated lipid hydroperoxides [9]. This procedure revealed that alkanes are formed continuously under physiological conditions [20]. Little is known, however, about the source of short-chain hydrocarbon products *in vivo*. Some investigators studied the exhalation of ethane and pentane in vitamin E-deficient rats and found an increase in the pentane concentration exhaled [21, 22]. It was suggested that the liver is probably the major source of volatile hydrocarbons associated with *in vivo* lipid peroxidation. Doubt arose when Burk and Lane [23] showed that many agents are capable of causing *in vivo* lipid peroxidation, as monitored by pentane release, but that lipid peroxidation does not always correlate with liver necrosis.

In the present study the *in vitro* and *in vivo* release of alkanes due to phenylhydrazine treatment of red blood cells and rats, respectively, was shown. *In vitro* we observed approximately equal amounts of ethane and pentane in contrast to *in vivo* experiments in which treatment of rats with phenylhydrazine resulted in a 6-fold increase in ethane and a 2-fold increase in pentane exhalation. This can be explained in particular by the metabolic elimination of some alkanes in the liver; e.g. pentane is known to be oxidized 5–8 times faster than ethane [18, 24].

Significant amounts of propane and *iso*-butane were produced from red cells as well as from haemoglobin alone during phenylhydrazine treatment, suggesting that these alkanes may originate from haemoglobin degradation and not from membrane lipid peroxidation. Further studies are in progress to clarify whether the release of propane may serve as an index of haemoglobin degradation due to free radical attack.

In conclusion, application of the described techniques provides a new means for the examination of the viability of red cells *in vivo* and *in vitro* which are known to be severely threatened under conditions of oxidative stress (i.e. induced by radiation, oxidative enzymes and drugs). An understanding of the mechanisms involved in haemolysis is particularly valuable for the investigation of pathogenesis in corpuscular haemolytic anaemias, e.g. glucose-6-phosphate dehydrogenase deficiency, for testing haemolytic drug effects and for determining red cell aging processes.

Acknowledgements—We thank M. Müh-Zange, U. Wegner and H. Einsele for technical assistance.

REFERENCES

1. O. Warburg, F. Kubowitz and W. Christian, *Biochem. Z.* **242**, 170 (1931).
2. S. K. Jain and P. Hochstein, *Archs Biochem. biophys.* **201**, 683 (1980).
3. H. P. Misra and I. Fridovich, *Biochemistry* **15**, 681 (1976).

4. A. Valenzuela, H. Rios and G. Neiman, *Experientia* **33**, 962 (1977).
5. S. K. Jain and P. Hochstein, *Biochim. biophys. Acta* **586**, 128 (1979).
6. G. Cohen and P. Hochstein, *Biochemistry* **3**, 895 (1964).
7. E. E. Dumelin and A. L. Tappel, *Lipids* **12**, 894 (1977).
8. U. Köster, D. Albrecht and H. Kappus, *Toxic. appl. Pharmac.* **41**, 639 (1977).
9. A. L. Tappel, in *Free Radicals in Biology* (Ed. W. A. Pryor), Vol. IV, 1–47. Academic Press, New York (1980).
10. A. Wendel and E. E. Dumelin, *Meth. Enzym.* **77**, 10 (1981).
11. C. A. Riely, G. Cohen and M. Lieberman, *Science* **183**, 208 (1974).
12. A. Müller, P. Graf, A. Wendel and H. Sies, *FEBS Lett.* **126**, 241 (1981).
13. H. Kappus and H. Muliawan, *Biochem. Pharmac.* **31**, 597 (1982).
14. R. Reiter and A. Wendel, *Biochem. Pharmac.* **32**, 665 (1983).
15. M. R. Clemens and H. Remmer, *Blut* **45**, 329 (1982).
16. E. Beutler, C. West and K. G. Blume, *J. Lab. clin. Med.* **88**, 328 (1976).
17. H. Frank, T. Hintze and H. Remmer, in *Applied Head Space Gas Chromatography* (Ed. B. Kolb), pp. 155–164. Heyden and Son, London (1980).
18. H. Frank, T. Hintze, D. Bimboes and H. Remmer, *Toxic. appl. Pharmac.* **56**, 337 (1980).
19. M. K. Longani and R. E. Davies, *Lipids* **15**, 485 (1980).
20. H. Remmer, T. Hintze, H. Frank and M. Müh-Zange, *Xenobiotica* (in press).
21. C. J. Dillard, E. E. Dumelin and A. L. Tappel, *Lipids* **12**, 109 (1977).
22. I. A. Herschberger and A. L. Tappel, *Lipids* **17**, 686 (1982).
23. R. F. Burk and J. M. Lane, *Toxic. appl. Pharmac.* **50**, 467 (1979).
24. J. G. Filser, H. M. Bolt, H. Muliawan and H. Kappus, *Archs Toxic.* **52**, 135 (1983).