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An Unusual Temperature Dependence of Malondialdehyde Formation in Fe^{2+}/H_2O_2-Initiated Lipid Peroxidation of Phosphatidylcholine Liposomes

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Summary

Determination of malondialdehyde is a widely used procedure for measurement of lipid peroxidation. In this paper we report an unusual temperature dependence of malondialdehyde formation in egg yolk phosphatidylcholine liposomes oxidized by the Fenton system (0.1 mmol/l $FeSO_4$ and 0.05 mmol/l H_2O_2). The amount of malondialdehyde formed was 37 % higher in samples kept at 22 °C than at 50 °C. An alternative method for determination of lipid peroxidation, measurement of oxygen uptake, revealed complete consumption of dissolved oxygen to peroxidized lipids at 22 °C as well as 50 °C. Since oxygen is essential for the formation of cyclic peroxides – precursors of malondialdehyde – we conclude that the nature of the observed effect consists in limitation of oxygen availability at elevated temperatures.

Key words

Lipid peroxidation – Malondialdehyde – Temperature dependence – Oxygen consumption

Introduction

Chemistry of lipid oxidative degradation has been studied in great detail and a variety of oxidation products of these reactions have been identified (Porter 1984, Frankel 1987). The decomposition product of cyclic peroxide compounds, malondialdehyde, is frequently used for determination of lipid peroxidation in different systems (Slater 1984, Mišík 1991a,b).

The present communication was prompted by our earlier finding that the extent of Fe^{2+}/ascorbate-induced lipid peroxidation in rat brain tissue, detected as malondialdehyde formation by the thiobarbituric acid method, was higher in samples incubated at room temperature compared to samples incubated at 50 °C (Alov, unpublished results). Due to the rather complex nature of this system, we were unable to clarify whether the nature of the observed effect was purely physico-chemical or whether the inactivation of some defense enzymatic systems at higher temperatures was involved.

In this paper we report and explain the nature of a similar unusual temperature dependence of malondialdehyde formation in a simple model system of phosphatidylcholine liposomes oxidized by the Fenton system (Fe^{2+}/H_2O_2).

Materials and Methods

Egg yolk phosphatidylcholine isolated as described elsewhere (Singleton *et al.* 1965) was kindly provided by Dr. Emil Švajdlenka (Comenius Univ. Bratislava), ferrous sulphate ($FeSO_4 . 7 H_2O$) was from Sigma (USA), hydrogen peroxide was from Lachema (Czech Republic), 2-thiobarbituric acid was from Fluka AG (Switzerland), malondialdehyde (bis (dimethyl) acetate) was from Merck (Germany).

Phosphatidylcholine liposomes were prepared as described elswhere (Ondriaš *et al.* 1989). Lipid peroxidation was initiated by addition of the Fenton system (Fe^{2+}/hydrogen peroxide) to the liposomal suspension to attain the final concentrations in 1 ml of

incubation solution (120 mmol/l KCl, 4 mmol/l Tris.HCl (pH 7.2), 5 mg lipid, 0.1 mmol/l $FeSO_4$ and 0.05 mmol/l H_2O_2. (Samples referred as autoxidized contained the same amount of lipids but the Fenton system was omitted.) Samples were incubated at the desired temperature in open vials with free access of air. Samples, in which the influence of temperature and of Fenton reagents was tested, instead of lipids contained 0.05 mmol/l of authentic malondialdehyde, prepared from malondialdehyde (bis dimethyl) acetate. At the desired intervals, 100 μl of the suspension were taken for determination of thiobarbituric acid-reactive substances (TBARS), mainly malondialdehyde (Haenen and Bast 1983). The malondialdehyde concentration was calculated from the absorbance at 533 nm (spectrometer Specord M 40, Karl Zeiss, Germany), assuming a molar absorption coefficient of 1.5×10^5 l.mol^{-1} cm^{-1} (Slater 1984).

Measurement of oxygen consumption: 5 ml of the liposomal suspension (5 mg of lipid/ml) was placed in the thermostated chamber and oxygen consumption was measured (Gergel' *et al.* 1992). Peroxidation was initiated by addition of the Fenton system (0.1 mmol/l $FeSO_4$ and 0.05 mmol/l H_2O_2). Oxygen uptake was measured using an Aquacheck-3 oxygen detector (Radelkis, Hungary) equipped with a Clark type oxygen electrode SOPS-31 (Chemoprojekt, Czech Republic).

Student's t-test was used to determine statistical significance of the results.

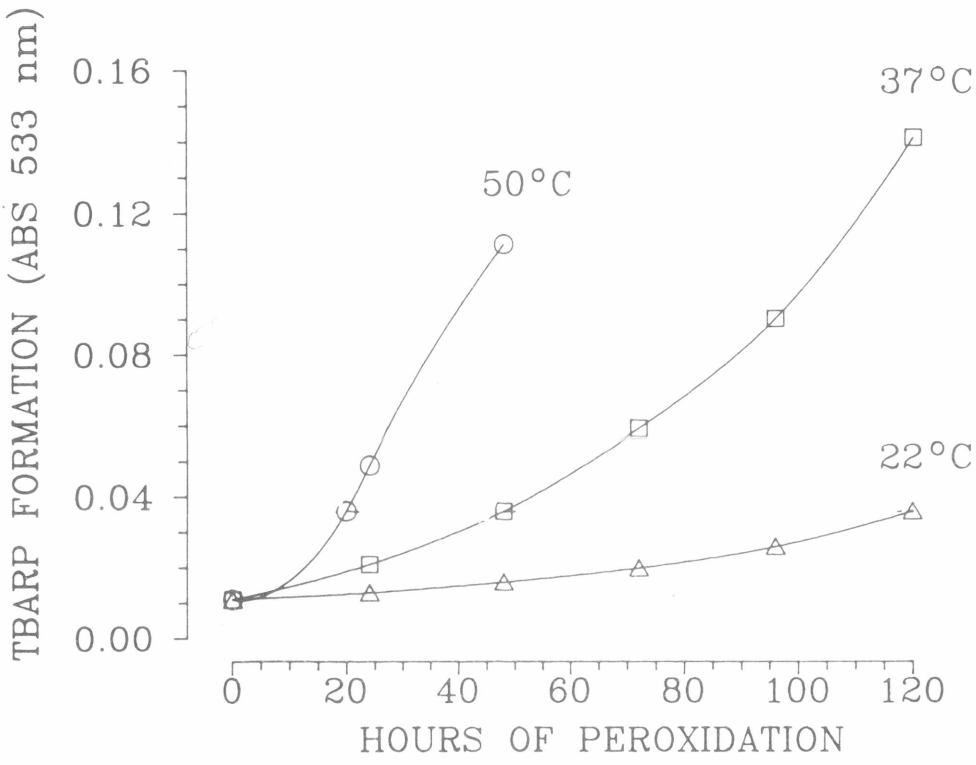

Fig. 1
Time courses of malondialdehyde formation (ABS 533 nm) during autoxidation of egg phosphatidylcholine liposomes kept at 50 °C (circles), 37 °C (squares), and 22 °C (triangles). Results are means from 3–4 determinations.

Results and Discussion

Time courses of malondialdehyde accumulation in autoxidized samples (without addition of the Fenton system) are depicted in Fig. 1. As expected, the rate of malondialdehyde accumulation was slow (typically several days before the maximum is reached) and rose with increasing temperature.

A very different picture was obtained when the Fenton system was used for initiation of lipid peroxidation (Fig. 2). Note that the rate of malondialdehyde formation in this system was much higher than in the absence of the Fenton system. After approx. 30 min of Fenton-initiated lipid peroxidation the malondialdehyde level in the samples kept at 22 °C, 37 °C, and 50 °C reached a maximum and its levels did not increase significantly during subsequent incubation. The most notable difference was, however, that this reaction had an apparent negative activation energy, i.e. the malondialdehyde production was inversely proportional to the incubation temperature, i.e. was highest at 22 °C and lowest at 50 °C.

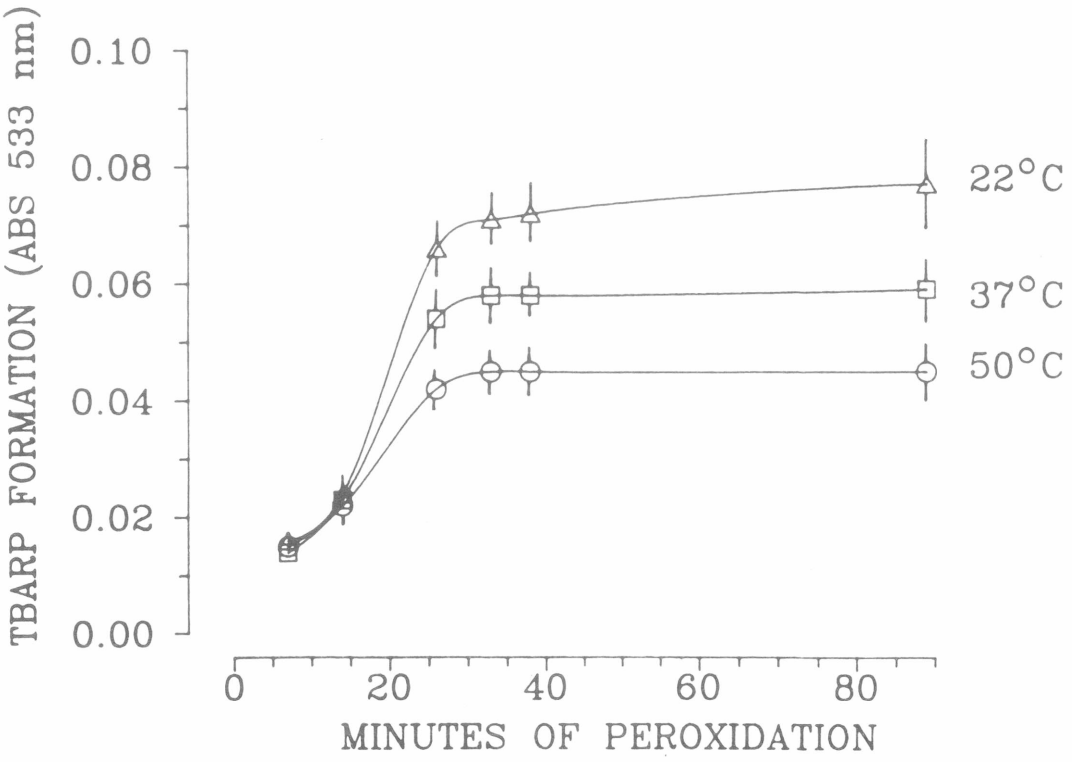

Fig. 2
Time courses of malondialdehyde formation (ABS 533 nm) in the Fenton system-initiated lipid peroxidation of egg phosphatidylcholine liposomes kept at 50 °C (circles), 37 °C (squares), and 22 °C (triangles). Results are means ± S.D. (n=4). The data at different temperatures were significantly different ($p < 0.02$) at all time points after 24 min.

Experiments with authentic malondialdehyde showed that neither the temperature of incubation nor the Fenton system affect the thiobarbituric acid-detectable malondialdehyde significantly (Table 1).

Table 1
Effect of sample temperature and the Fenton reagents on the thiobarbituric acid-detectable (ABS 533 nm) authentic malondialdehyde (MDA).

MDA	22 °C	0.624±0.009
MDA	50 °C	0.633±0.037
MDA + Fe^{2+}/H_2O_2	22 °C	0.612±0.010
MDA + Fe^{2+}/H_2O_2	50 °C	0.651±0.044

An alternative method was also used for the determination of lipid peroxidation. Measurement of oxygen uptake in the peroxidized samples in the early stages of lipid peroxidation follows the peroxide formation (Dahle *et al.* 1962). Using this method we observed a complete consumption of dissolved oxygen at 22 °C as well as at 50 °C (Fig. 3). Depicted traces of oxygen uptake are representative from four experiments. In each case complete oxygen consumption (to 2–8 % of its starting value) was observed within 20 min after the addition of the Fenton system to the liposomal suspension. Less than 10 % of dissolved oxygen was consumed in samples containing either Fe^{2+} alone or the complete Fenton system (Fe^{2+} and H_2O_2) in the absence of lipids (not shown). These experiments confirmed that the consumed oxygen was taken up by oxidizing lipids and not by autoxidizing Fe^{2+} present in the system (Kosaka *et al.* 1992).

Relative values of dissolved oxygen measured by the Clark electrode were expressed in μmoles by extrapolation of tabulated values (Hitchman 1978). Due to the increase of oxygen solubility with decreasing temperature there was a higher starting value of dissolved oxygen at 22 °C than at 50 °C. Thus, the total amount of oxygen consumed was higher in samples kept at 22 °C than at 50 °C. We observed complete oxygen uptake even in samples under free access of air, as the rate of oxygen uptake to the peroxidized lipids was at least 20 times higher than the rate of oxygen diffusion from air to the samples (not shown).

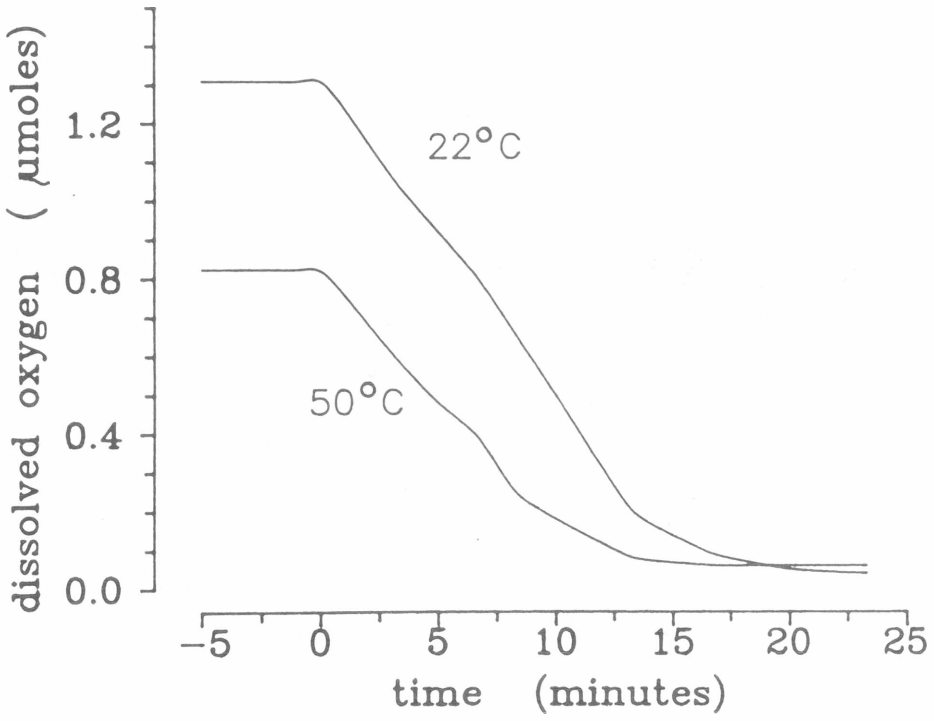

Fig. 3
Representative time courses of oxygen uptake to egg phosphatidylcholine liposomes peroxidized by the Fenton system at 22 °C and 50 °C. Fenton system was added at time 0.

Under such conditions, the concentration of oxygen in the oxidizing system decreases to such an extent that the rate-limiting step of lipid peroxidation chain reactions is not the propagation reaction (2), but rather the diffusion controlled reaction (1) (Emanuel *et al.* 1965):

$$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot} \quad (1)$$
$$LOO^{\cdot} + LH \longrightarrow LOOH + L^{\cdot} \quad (2)$$

Finally there is no more oxygen available and reaction (1) does not take place. As a result, lipid hydroperoxides cannot be formed in reaction (2). Accordingly, the amount of lipid hydroperoxides created under such conditions must be directly related to the total amount of available oxygen. Since the malondialdehyde is formed by decomposition of cyclic hydroperoxides (Porter 1984, Frankel 1987, Dahle *et al.* 1962, Pryor *et al.* 1976) we expect that the malondialdehyde production is directly related to the amount of the consumed oxygen. If so, then the ratio of malondialdehyde created at 22 °C and 50 °C should be equal to the ratio of total oxygen dissolved in the samples (provided that there is no reuptake of oxygen from the air).

Therefore, the malondialdehyde content in the samples was evaluated after completion of oxygen uptake measurements. A good agreement was obtained between the ratio of malondialdehyde content at 22 °C and 50 °C (1.65±0.04, n=4) and the ratio of nanomoles of oxygen dissolved at these temperatures (1.59, calculated from Hitchman, 1978). Oxygen consumption per nanomol of malondialdehyde formed at 22 °C and 50 °C also showed an excellent agreement (10.4±0.3 and 9.9±0.8 nmol). Slightly higher values of oxygen consumed per malondialdehyde formed were reported by others (Wills 1969). This difference can be attributed to the different fatty acid composition of the lipids used.

Thus, we conclude that the observed unusual temperature dependence of malondialdehyde formation was due to the limitation in oxygen availability, caused by a higher rate of oxygen consumption than its diffusion from the air. Thus malondialdehyde formation in this rapidly oxidizing system was limited by the amount of dissolved oxygen which is higher at 22 °C than at 50 °C. Similar

behaviour would be expected in all rapidly oxidizing systems (such as the Fenton system, iron/ascorbate, or azo-compound initiated peroxidation) in which oxygen uptake into peroxidized lipids is higher than its spontaneous diffusion from the surrounding medium.

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