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NADPH-INDUCED MICROSOMAL LIPID PEROXIDATION AS MEASURED BY MALONDIALDEHYDE PRODUCTION IN RAT LIVER. INHIBITORY EFFECT OF NAFTIDROFURYL

ROSA PARUSSINI† and CATHERINE GODINOT

LBTM - CNRS, Université Claude Bernard Lyon I 43, boulevard du 11 novembre 1918–69622 Villeurbanne Cedex, France

DANIEL GUERRIER

Centre de recherche LIPHA, B. P. 8481 - 69359 LYON CEDEX 08, France

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The effects of naftidrofuryl have been studied on NADPH-induced microsomal lipid peroxidation by measuring malondialdehyde (MDA) production. Conditions adequate to measure MDA production and effects of naftidrofuryl on MDA production have been tested. It has been shown that the addition of ferric ions is essential with Tris or Pipes buffers while it can be omitted with phosphate known to contain traces of ferric ions. However the initial rate of MDA production is much lower with phosphate in the absence of added ferric ions, showing that the initiation of lipid peroxidation is limited by ferric ions. The effects of naftidrofuryl have been studied on MDA production in phosphate buffer in the presence or absence of ferric ions. Naftidrofuryl inhibits lipid peroxidation in both conditions indicating that the inhibition is not related to an interaction with added ferric ions. Naftidrofuryl is efficient at concentrations slighty higher than butylhydroxytoluene but lower than aminopyrine.

Key words: Lipid peroxidation; malondialdehyde; naftidrofuryl; microsomes; rat liver.

Abbreviations: (MDA): malondialdehyde; (LS 121): naftidrofuryl salt oxalate; (BHT): butylhydroxytoluene; (pHMB): parahydroxymercuribenzoate; (EDTA): ethylenediaminetetracetic acid; (Tris): tris [hydroxymethyl]-aminomethan; (Pipes): piperazine-N, N'-bis [2-ethane-sulfonic acid];

INTRODUCTION

Naftidrofuryl has been reported to exert a protective effect against hypobaric

† To whom correspondence should be addressed.

hypoxia[1] and against the metabolic damages of ischemia[2]. Demopoulos et al[3] have shown that ischemia causes pathologic lipid free radical reactions presumably by producing lipid hydroperoxides. Similarly, ageing increases the amount of superoxide radicals[4]. In this study we have tested if the benefical effects of naftidrofuryl in hypoxia, ischemia or ageing could be related to a direct inhibitory effect on lipid peroxidation. Since naftidrofuryl induces vasodilatation[5], it was important to use an '*in vitro*' model independent of blood circulation. The NADPH-induced microsomal lipid peroxidation has been chosen for this study[6]. The microsomal fraction can catalyze an NADPH-induced peroxidation of endogenous lipids in presence of ferric ions[6]. This reaction leads to the transient formation of phospholipid peroxides which promote the breakdown of polyunsatured fatty acids and produce a variety of degradation products such as malondialdehyde (MDA)[7].

Since the experimental conditions used to test the ability of microsomal fractions to produce MDA vary from one laboratory to the other, we have first compared various procedures in order to determine conditions adequate to study the effects of naftidrofuryl.

It will be shown that naftidrofuryl effectively inhibits MDA production in rat liver microsomal fraction either in the presence or the absence of added ferric ions.

MATERIALS AND METHODS

Preparation of microsomes

Wistar rats (IUT of Biology, LYON I) weighing 200 to 350g, fed ad libitum, were used to prepare hepatic microsomal fraction by a method modified from[8]. The livers, washed with 0.154 M NaCl, 10 mM Tris-HCl, pH 7.4 were homogenized in buffer A (0.09 M NaCl, 0.05 M sodium phosphate, pH 7.4) The supernatant fraction obtained after the first centrifugation (2 700 g × 10 min) was filtered on cheese cloth and centrifuged at 30 000 g for 10 min. The new supernatant fraction was filtered again and centrifuged at 100.000 g for 30 min.[8] Under these conditions, no cytochrome c oxydase[9] or succinate cytochrome c reductase activity[10] could be detected in the microsomal pellet, indicating the absence of mitochondrial markers. The microsomal fraction contained a typical NADPH-cytochrome c reductase activity[10] of 84 μmoles cytochrome c reduced/min/g protein and a cytochrome P 450[11] content of 0.47 μmoles/g protein. The protein concentration was estimated according to[12]. Microsomal pellets were kept frozen in liquid nitrogen.

Determination of MDA formation

Microsomal pellets were thawed and homogenized in buffer A (4°C) at a concentration of about 10 mg protein/ml. They were preincubated for 5 min at 38°C in a shaking water bath with the indicated buffer at a protein concentration of 0.2–0.6 mg/ml. It has been checked that, under these conditions, the MDA production was proportional to protein concentration. The reaction was initiated by addition of 0.1 mM NADPH and stopped by adding 1 ml of 20% trichloracetic acid in 0.6 N HCl (TCA) per 1 ml of incubation medium. After centrifugation at 5000 g for 15 min, the MDA content of the supernatant fraction was determined at 535 nm by the thio-barbituric acid reaction[13] using a molar extinction coeficient of 1.56×10^{-5} M^{-1}

cm^{-1}. It was checked that none of reagents used in this study interfered with MDA determination and that no modification was observed in the rate of MDA production after freezing of microsomal pellets.

RESULTS AND DISCUSSION

Optimal conditions to estimate the rate of NADPH-induced lipid peroxidation by the determination of MDA production

Although the lipid peroxidation of microsomes has been correlated with MDA formation[7, 14], the experimental conditions used to produce MDA are somewhat different in various reports. Roders et al[8] observed MDA formation in phosphate buffer, pH 6.6 in the absence of added iron. Poyer and Mc Cay[15] have shown that MDA production was dependent on added ferric ions but that the iron present as a contaminant in phosphate buffers was sufficient and that the presence of a complex 'ADP-Fe' was mandatory in iron-free phosphate or Tris buffers. Kornburst and Mavis[16] used Tris-maleate, pH 7.4 and ferric ions without ADP, the lipid peroxidation being initiated by addition of NADPH and a xanthine-oxidase system.

Figure 1 shows the effects of various buffers and of the presence of 'ADP-Fe' on MDA production. In the absence of 'ADP-Fe' the MDA production can only be demonstrated in phosphate buffer. In the other buffers tested, Tris or Pipes, MDA formation tends to slightly decrease from pH 6.5 to 7.5 when 'ADP-Fe' is present. On the contrary, in the presence of 'ADP-Fe', the MDA production in phosphate buffer slightly increased from pH 6.4 to 7.5. In the presence of 'ADP-Fe', the MDA production is about 2-fold higher in Tris or Pipes buffers than in the phosphate buffer. The phosphate-induced inhibition was independent of the ionic strength from 0 to 180 mM NaCl, in the presence of 10 mM Tris-HCl, pH 7.4 (not shown). The inhibitory effect of phosphate observed here is in agreement with that observed when lipid peroxidation was initiated by both NADPH and the xanthine-oxidase system[16].

Figure 2 shows that the kinetics of MDA formation is very different in the presence or absence of 'ADP-Fe'. In presence of 'ADP-Fe', the MDA production is biphasic: the first rapid phase slows down after 10 min and is barely dependent upon NADPH concentration while the rate of the second phase increases with NADPH concentration. In the absence of 'ADP-Fe', the formation of MDA starts slowly and then increases linearly up to 40 min, when a plateau is reached. It is generally admitted that lipid-peroxidation occurs in two sequential steps: initiation and propagation[17]. Our experiments are in agreement with the hypothesis suggesting that iron or 'ADP-Fe' complex stimulates the initiation step of lipid peroxidation[17]. The difference in the kinetics observed in the presence or absence of 'ADP-Fe' suggests that the limiting step in the first phase of MDA production in the absence of 'ADP-Fe' may be related to the level of iron while the limiting step in the presence of 'ADP-Fe' is of a different nature. The effect of naftidrofuryl has been studied in both conditions to see if the naftidrofuryl effects could be related to the level of iron.

Effect of naftidrofuryl on MDA production

Table I shows that naftidrofuryl complexed with oxalate inhibits MDA production while oxalate has no effect. The concentration of naftidrofuryl inducing 50%

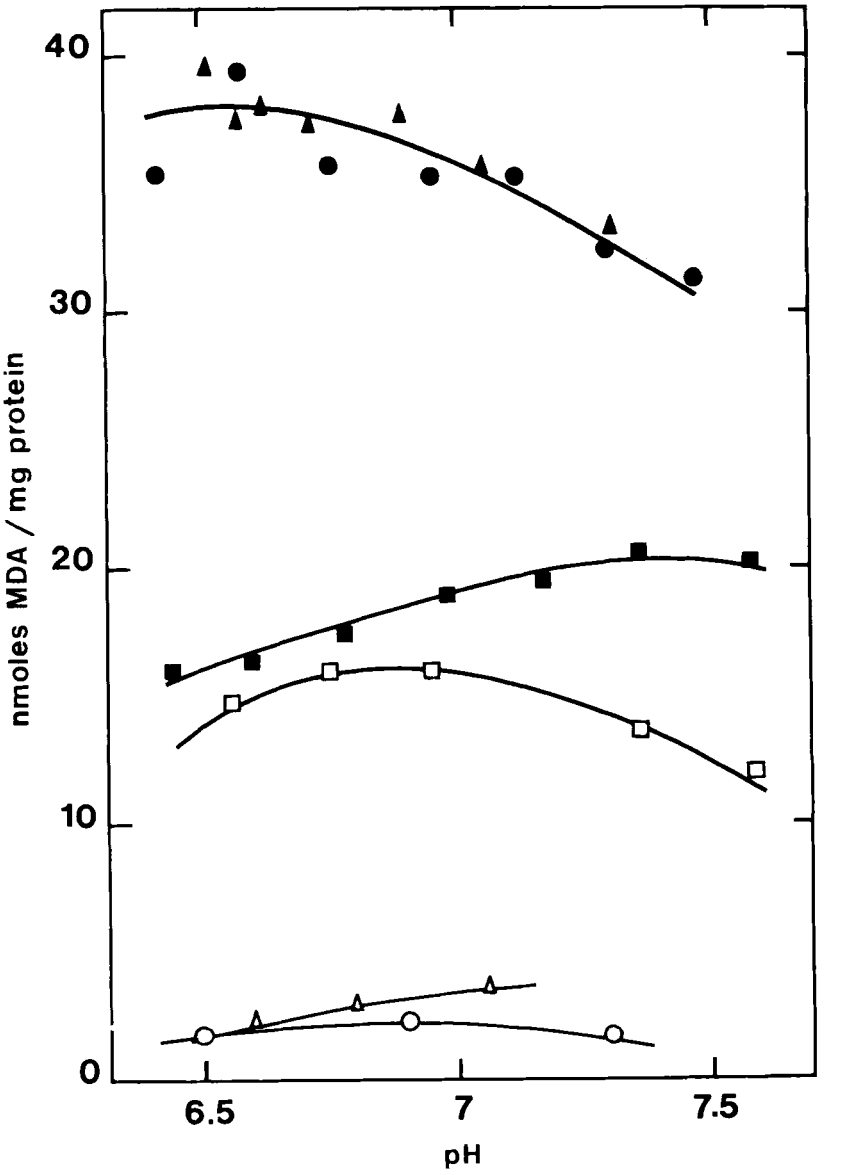

FIGURE 1: Dependence of MDA accumulation in 20 minutes on buffer, pH and presence of 'ADP-Fe'. A sample (50 μl) of the microsomal fraction homogenized in 0.09 M NaCl, 0.05 M sodium phosphate, pH 7.4 was preincubated 5 min at 38°C in 1.25 ml (final volume) of the buffer tested. The protein concentration was 0.36 mg/ml. The reaction was initiated by adding 0.1 mM NADPH and stopped 20 min later by TCA addition. MDA formation was estimated as indicated in Material and Methods. All buffers contained 0.09 M NaCl and: (△,▲) 0.05 M Tris-HCl; (○,●) 0.05 M Pipes; (□,■) 0.05 M sodium phosphate. Empty symbols: no 'ADP-Fe' added. Closed symbols: 1.7 mM ADP + 0.1mM $FeCl_3$. In all cases the indicated pH was obtained by addition of either HCl or NaOH.

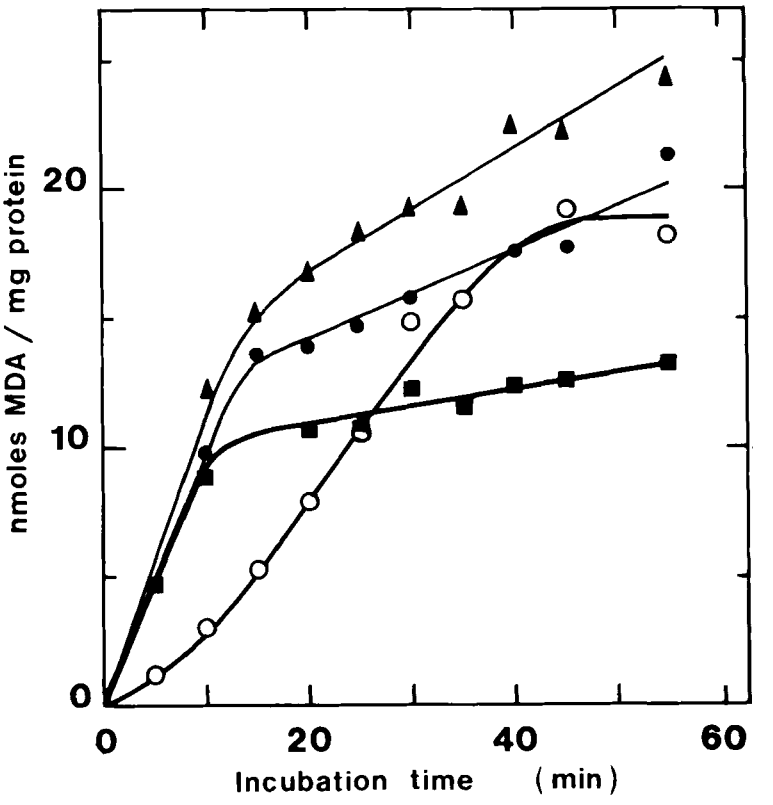

FIGURE 2: Kinetics of MDA production in the presence of various NADPH concentrations and in the presence or absence of 'ADP-Fe'. The production of MDA was estimated as described in Fig. 1, in 0.09 M NaCl-0.05 M sodium phosphate, pH 7.4. The protein concentration was 0.29 mg/ml. The reaction was started by adding respectively 0.1 mM NADPH (■); 0.3 mM NADPH (○,●) or 0.1 mM NADPH with a regenerating system (▲) containing: glucose 6 phosphate 5 mM + glucose 6 phosphate dehydrogenase 1.7 unit. All systems contained 'ADP-Fe' (1.7 mM ADP + 0.1 mM $FeCl_3$) except for the curve (○).

inhibition was of about 55 μM in the presence of 'ADP-Fe' and of about 25 μM in the absence of 'ADP-Fe'.

The inhibitory effect observed in the absence or presence of 'ADP-Fe' is not significantly different. The kinetics of MDA production in the presence of naftidrofuryl inducing a 50% inhibition was parallel to that of the control whether 'ADP-Fe' was present or not (not shown). Since there is no difference in the two systems and since at 10 min, the initiation step of the reaction was more limited in the absence of 'ADP-Fe' (see fig. 2), it is likely that naftidrofuryl does not interfere with the initiation step but rather in the propagation step of the lipid peroxidation, that is during the production of lipid peroxides and malondialdehyde[17, 18]. The inhibitory effect of naftidrofuryl is independent of the time of preincubation of naftidrofuryl with the microsomal fraction (0 to 90 min). Therefore, the drug does not have to be metabolized to be active. Besides, the inhibition was identical whatever the NADPH concentration was (0.025 to 0.5 mM, in the presence or absence of a NADPH-

regenerating system) (not shown). Therefore naftidrofuryl does not seem to compete with NADPH.

The comparison of the effects of naftidrofuryl to those of other known inhibitors of lipid peroxidation (Table I) further indicates that this drug should not directly interfere with ferric ions. We have tested 3 inhibitors which do not directly interfere with ferric ions: the butylhydroxytoluene (BHT) which is an antioxidant[18], aminopyrine which acts by competition with the NADPH-cytochrome c reductase[19] involved in the microsomal lipid peroxidation[20] and the parahydroxymercuribenzoate (pHMB) which acts on sulfhydryl groups of the microsomal electron transport enzymes[21]. These inhibitors decrease similarly the MDA accumulation in the presence or in the absence of 'ADP-Fe' as does the naftidrofuryl. On the contrary, the naftidrofuryl has a completely different pattern of inhibition from that of EDTA which acts directly on ferric ions present in the incubation medium. EDTA is a very potent inhibitor of MDA production in the absence of 'ADP-Fe' while it activates or inhibits MDA production in the presence of 'ADP-Fe' depending upon the relative concentrations of EDTA and Fe in agreement with previous observations[22].

In conclusion, naftidrofuryl is a potent inhibitor of lipid peroxidation and it acts independently of the level of iron and of NADPH in the incubation medium. Further work is in progress to test if this drug can also have a protective effect against lipid peroxidation '*in vivo*', especially in the brain.

TABLE I
Effects of various inhibitors on MDA production.

Addition	10^{-3}M	10^{-4}M	10^{-5}M	10^{-6}M
LS 121 + ADP-fer (8)	9.3 ± 2.3	44.9 ± 5.0	70.8 ± 3.8	86.3 ± 3.6
– ADP-fer (7)	8.3 ± 2.5	38.6 ± 6.1	62.6 ± 8.9	75.4 ± 7.0
Oxalate + ADP-fer (8)	102.4 ± 3.0	–	–	–
– ADP-fer (7)	106.4 ± 3.6	–	–	–
EDTA + ADP-fer (4)	7.0 ± 2.7	8.4 ± 2.5	159.2 ± 17.9	127.8 ± 11.3
– ADP-fer (4)	11.6 ± 5.4	8.5 ± 5.7	24.7 ± 5.8	101.2 ± 9.1
BHT + ADP-fer (4)	–	3.0 ± 1.5	9.5 ± 6.0	97.3 ± 10.6
– ADP-fer (4)	–	1.0 ± 0.6	2.5 ± 1.9	86.2 ± 13.7
pHMB + ADP-fer (5)	–	9.2 ± 3.6	87.8 ± 5.3	84.3 ± 9.7
– ADP-fer (5)	–	17.8 ± 9.0	93.6 ± 12.6	69.0 ± 16.2
Aminopyrine + ADP-fer (3)	60.3 ± 3.9	99.3 ± 7.0	–	–
– ADP-fer (3)	50.3 ± 4.3	107.7 ± 10.3	–	–

Results are expressed as % of the controls made in the absence of inhibitor + S.E. The MDA produced in 10 min was 11.3 ± 0.9 nmoles of MDA/g of protein for controls containing 'ADP-Fe' and 7.8 ± 1.2 nmoles of MDA/g for controls in the absence of 'ADP-Fe' (mean of 13 experiments + S.E.). The incubation was carried out as described in Material and Methods in 0.09 M NaCl, 0.05 M sodium phosphate, pH 7.4. The inhibitor was present in the preincubation medium at the indicated concentration. The number of experiments is indicated in parenthesis.

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