Biochemical Pharmacology, Vol. 41, No. 5, pp. 709–714, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

THE INHIBITION OF LIPID PEROXIDATION BY CINNARIZINE

POSSIBLE IMPLICATIONS TO ITS THERAPEUTIC AND SIDE-EFFECTS

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(*Received* 5 *January* 1990; *accepted* 23 *September* 1990)

Abstract—Cinnarizine has antivasoconstrictor properties and improves red-cell deformability. Its major side-effects are the induction of extrapyramidal reactions. It is a calcium antagonist, but it was suggested that its effects may depend on other mechanisms, namely on antiperoxidant properties. We have studied these properties in different biological systems, intact red-cells included. The occurrence of lipid peroxidation was determined by the formation of 2-thiobarbituric acid reactive products. Cinnarizine was found to inhibit spontaneous lipid peroxidation in rat liver homogenates, copper-induced lipid peroxidation in human plasma and copper-induced and hydrogen peroxide-induced lipid peroxidation in human red-cells. In red-cells, the inhibition of lipid peroxidation is accompanied by the inhibition of hemolysis. Copper-induced red-cell lipid peroxidation is 85% inhibited by as little as 5 μM cinnarizine. The antioxidant activity of cinnarizine may contribute to explain some of the effects of this drug.

Cinnarizine is a piperazine derivative first synthesized in 1958 as an antihistaminic. Later on it was found to be a labyrinthine sedative as well as a peripheral antivasoconstrictor. It was also suggested that it preserves erythrocyte deformability and protects cells during ischemia. The antivasoconstrictor effect depends on its selective calcium entry blocker properties. The other above-mentioned actions may also be attributed, perhaps although not exclusively, to calcium antagonism [1–5].

On the basis of these properties and of possible psychotropic actions, cinnarizine has been widely prescribed in certain countries, mainly for old people [6]. Major side effects for this drug were not reported until Martí Massó *et al.* [7] suggested a causal relationship between the use of cinnarizine and Parkinsonism.

Janero *et al.* [8] have recently reported that some calcium antagonists, cinnarizine included, exert membrane-protective effects as antiperoxidants and suggest this property may contribute to their pharmacologic actions. These conclusions were taken from studies using isolated rat myocardial phospholipids which were exposed to an oxygen radical producing system with xanthine oxidase and iron.

The present experiments were designed to test if the calcium antagonist cinnarizine also proves to have antiperoxidant properties in other biological products, intact cells included, exposed to some other oxidant systems. Cinnarizine was added to rat liver homogenate, to human plasma and to human red-cells. Lipid peroxidation was evaluated by the appearance of 2-thiobarbituric acid reactive products (TBArp). We show cinnarizine inhibits spontaneous, copper-induced and hydrogen peroxide-induced lipid peroxidation. In red-cells it inhibits lipid peroxidation and also the subsequent hemolysis.

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MATERIALS AND METHODS

Female Sprague–Dawley rats (250–300 g) with free access to a standard laboratory diet were killed by drawing blood by cardiac puncture after being anesthetized briefly with diethyl ether. The liver was perfused *in situ* with cold physiological saline to remove as much blood as possible. The major liver lobe was homogenized in a teflon pestle–glass homogenizer in 150 mM KCl at 4°. The homogenate was used immediately after preparation. The incubation mixtures included 0.5 mL of 15% homogenate (w/v), 0.1 mL of 150 mM KCl, ethanol or cinnarizine in ethanol and 150 mM phosphate buffer (pH 7.4 at 37°) to a final volume of 2 mL. They were placed in Erlenmeyer flasks and incubated for 45 min in a shaking water bath at 37° under air and in the dark. The presence of TBArp was determined as described by Ohkawa *et al.* [9] and used as an indicator of lipid peroxidation.

Plasma and erythrocytes were isolated by centrifugation from fresh heparinized blood from healthy donors. Erythrocytes were washed three times with ice-cold physiological saline. Red-cell suspensions in physiological saline adjusted to a final concentration in the assay of 5% (v/v) were prepared and immediately used. These suspensions were preincubated with 2 mM sodium azide for 1 hr at 37° in a shaking water bath. Sodium azide inhibits catalase and so potentiates oxidative stress in red-cells [10, 11]. Plasma was also diluted with saline to a final concentration in the assay of 5% (v/v) and immediately used.

Table 1. The effect of cinnarizine on lipid peroxidation in rat liver homogenates incubated in an atmosphere of air (incubation time: 45 min)

Additions	TBA value ($A_{532\,nm} \times 10^3$)	
	No incubation	45 min incubation
None	79.1 ± 31.0	212.3 ± 94.0 (1)
Ethanol 856 mM	70.7 ± 38.8	187.9 ± 93.7 (2)
Cinnarizine 25 μM	—	177.4 ± 95.1 (3)
Cinnarizine 250 μM	—	133.3 ± 74.1 (4)
Cinnarizine 1000 μM	68.0 ± 40.7	97.6 ± 48.3 (5)

Cinnarizine was added in ethanol.
Mean values ± SD are given (N = 7).
The following P values were calculated using Student's *t*-test for paired samples: (2) vs (1) $P < 0.001$; (3) vs (2) $P < 0.01$; (4) vs (2) $P < 0.001$; (5) vs (2) $P < 0.01$.

Copper chloride in distilled water (0.08 mL) and ethanol and cinnarizine solutions in ethanol (0.02 mL) were added to red-cell suspensions or to diluted plasma as stated. Copper was added 5 min after cinnarizine. The final volume of the mixture was 5 mL. The incubations were carried out in 25-mL Erlenmeyer flasks, in a shaking water bath at 37° under air and in the dark.

The red-cell suspensions were also exposed to hydrogen peroxide, reproducing the "addition technique" described by Stocks and Dormandy [10]. In these experiments the final volume in each flask was 10 mL and cinnarizine in ethanol was added as 0.1-mL aliquots.

The presence of the TBArp was determined as previously described by some of us [11, 12]. We name "TBA value" the product by 10^3 of the absorbance values given by the TBA tests. Methemoglobin formation and hemolysis were determined by direct spectrophotometrical methods [13].

Chemicals. All reagents were obtained in the maximum degree of purity available and used as supplied. Cinnarizine was a gift from Janssen Laboratories. Sodium azide and Triton X-100 were from Sigma. Cupric chloride ($CuCl_2\ 2H_2O$) and *N*-butyl alcohol were from May and Baker. Acetic acid, glacial, was from BDH. All other chemicals, including 2-thiobarbituric acid and the stock solution of 30% hydrogen peroxide (w/v), were products of Merck.

RESULTS

Experiments with liver homogenates

As shown in Table 1, when liver homogenates are incubated in an atmosphere of air, TBArp formation occurs. The observed increase was of 169.4 ± 80.1%. Ethanol, the solvent used for cinnarizine, inhibits this increase a little (13.0 ± 5.1%) when present at a concentration of 856 mM. Cinnarizine, in much smaller concentrations, has a clear additional inhibitory effect: 47.0 ± 12.4% for 1 mM cinnarizine and 30.2 ± 5.3% for 0.25 mM cinnarizine.

We have observed that cinnarizine does not directly interfere with the employed method for TBArp determination: when cinnarizine is added to homogenates after the incubation step there is no significant change in the TBA value.

Effect of cinnarizine on copper-induced lipid peroxidation in plasma and in red blood cells

At the end of a 3 hr incubation period of diluted plasma under air the TBA value is 6 ± 4. The presence of 30–500 μM cinnarizine in ethanol during the incubation does not alter this value. If 125 μM copper chloride is present during the incubation there is a marked stimulation of lipid peroxidation, with an increase of the TBA value to 695 ± 209 (N = 21).

With red blood cells a similar effect is observed: the presence of 125 μM copper chloride during the 75 min incubation determines an increase of the TBA value from 11 ± 5 to 378 ± 134 (N = 12). The presence of 30–500 μM cinnarizine in ethanol during the incubation without copper does not alter the basal value.

The addition of copper chloride only after the incubation steps to a final concentration of 125 μM, just before the samples are submitted to the TBA method, does not change the basal TBA values. The presence of ethanol (the solvent for cinnarizine), in addition to copper, during the incubation steps does not alter the copper effect in plasma over ±2.2% and in erythrocytes over ±3.0%.

Cinnarizine inhibits copper-induced lipid peroxidation both in pasma (Fig. 1) and in erythrocytes (Fig. 2). With all the cinnarizine concentrations used, with the exception of 1 μM cinnarizine in plasma, the effect was always inhibitory although the intensity of the inhibitory action varies considerably with plasma or with red blood cells from different donors. The smaller cinnarizine concentrations were more effective in inhibiting red-cell lipid peroxidation than plasma lipid peroxidation.

Cinnarizine in concentrations under 50 μM has a minimal hemolytic effect on the red blood cell suspensions: the hemolysis observed after 75 min of incubation was inferior to 2.8%. With higher concentrations of cinnarizine, hemolysis increases: 350 μM cinnarizine may induce an hemolysis as high

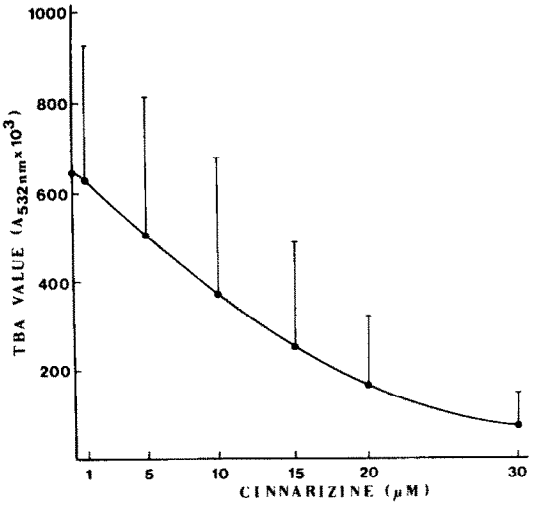

Fig. 1. Cinnarizine effect on 125 μM copper-induced lipid peroxidation in diluted plasma. Incubation time: 3 hr. Data are given as means ± SD, N = 6 (with plasma from different donors). Increasing cinnarizine concentrations in all the assayed plasma cause increasing inhibition of lipid peroxidation.

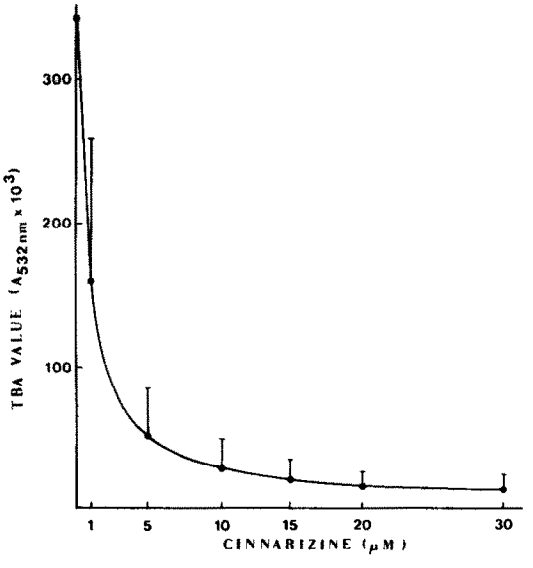

Fig. 2. Cinnarizine effect on 125 μM copper-induced lipid peroxidation in red blood cells. Incubation time: 75 min. Data are given as means ± SD, N = 7 (with red blood cells from different donors). Increasing cinnarizine concentrations in all the assayed red blood cell samples cause increasing inhibition of lipid peroxidation.

as 24.5%. Cinnarizine did not induce methemoglobin formation.

The time-course of red-cell lipid peroxidation and of hemolysis in the presence of copper and of copper plus cinnarizine is shown in Fig. 3. Evidence of the occurrence of lipid peroxidation precedes the hemolysis in both instances. In the presence of cinnarizine there is a slower rate of peroxidation and a great reduction of the extent of peroxidation. This inhibition of peroxidation is accompanied by an inhibition of hemolysis.

Effect of cinnarizine on hydrogen peroxide-induced lipid peroxidation and hemolysis

The exposure of red-cells to hydrogen peroxide induces auto-oxidation of red-cell lipids and hemolysis, as was shown by Stocks and Dormandy [10] and reproduced by us (Fig. 4). Ethanol, which is the solvent for cinnarizine, did not interfere with the hydrogen peroxide effects when present at a final concentration of 171 mM. Cinnarizine had an inhibitory effect both on lipid peroxidation and on hemolysis. This effect is already verified with 5 μM cinnarizine.

DISCUSSION

The inhibition of lipid peroxidation by cinnarizine seems to be independent of the oxidant system used to induce the peroxidation, as it was verified when using xanthine oxidase and iron [8], as well as simple exposure to air (for liver homogenates), copper (for plasma and red-cells) or hydrogen peroxide (for red cells). So, it is more likely that cinnarizine interferes with lipid peroxidation initiation or propagation but not with the components of the oxidant systems by inhibiting the enzymes or by scavenging the transitional metals.

As calcium increases lipid peroxidation in red-cells [14], the calcium-entry-blocking-action of cinnarizine could be implicated in the inhibition of red-cell lipid peroxidation. This cannot be the case in our assay system as there is no calcium in the incubation medium.

The antiperoxidant effect on red-cells which we have verified may be relevant, because it proves cinnarizine may have this type of effect on intact cells and because it may be one of the mechanisms of protection of red-cell deformability. Cinnarizine has this inhibitory effect at very low concentrations when compared with those of other agents we have tested before (like ascorbic acid, vitamin E, formate, benzoate and manitol) [11].

It is known that erythrocytes are susceptible to the oxidative stress [15, 16] and that the erythrocyte membrane phospholipid peroxidation may interfere with red-cell deformability [17, 18]. A close relationship between ischemic-reperfusion cell injury, calcium, oxidative stress and lipid peroxidation was postulated [19–22]. Cinnarizine being both a calcium-entry-blocking agent and an inhibitor of lipid peroxidation could interfere with these proposed pathogenic mechanisms. Some studies have suggested that cinnarizine lowers blood viscosity in patients with vascular diseases by improving red-cell deformability [2, 23].

Cinnarizine, like flunarizine—a difluorinated derivative of cinnarizine—may induce extra-pyramidal reactions [24] and aggravates Parkinson's

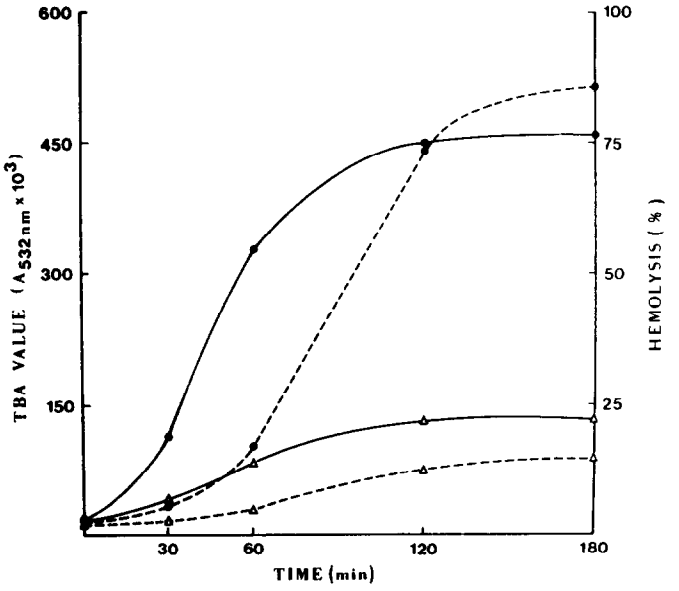

Fig. 3. Effect of 5 μM cinnarizine (△) on the kinetics of red-cell lipid peroxidation (——) and hemolysis (– – –) induced by 125 μM copper chloride. The values shown are from a representative of four experiments.

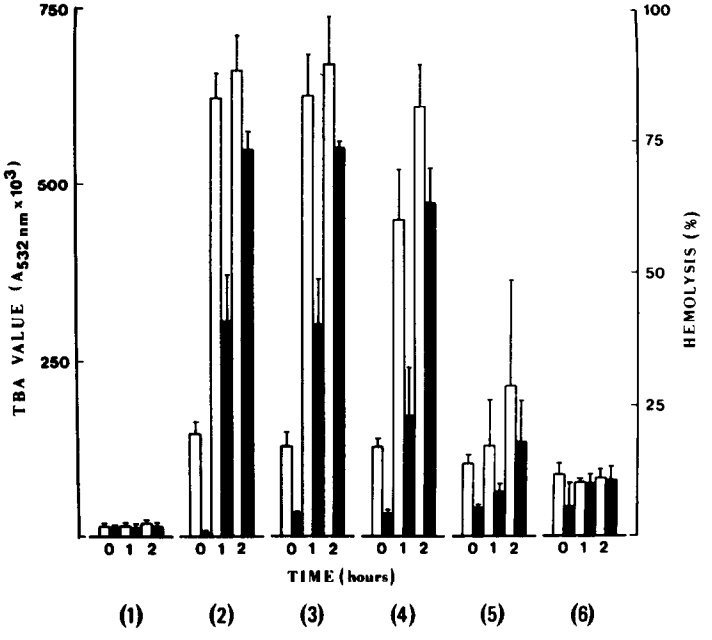

Fig. 4. Kinetic and concentration dependence study of the effects of cinnarizine in red-cell hydrogen peroxide induced lipid peroxidation and hemolysis. TBArp (□) and hemolysis (■) determinations were done before the incubation and at 1 and 2 hr of incubation: (1) red-cell suspension only; (2) red-cell suspension with 10 mM hydrogen peroxide: (3) red-cell suspension with 10 mM hydrogen peroxide and 171 mM ethanol; (4) as (3) plus 5 μM cinnarizine; (5) as (3) plus 25 μM cinnarizine; (6) as (3) plus 50 μM cinnarizine. Data are given as means ± SD, N = 4 (with red blood cells from different donors).

disease [25]. The precise mechanisms of these effects remain unknown.

Although one of the theories for Parkinson's disease pathogenesis links oxygen free radicals and lipid peroxidation to cell destruction in the substancia nigra [26–29], cinnarizine does not seem able to induce an oxidative stress. On the contrary, it inhibits lipid peroxidation in all the assayed systems and

some other calcium antagonists, flunarizine included, have proved to have antiperoxidant properties in brain microsomal membranes [30].

Cinnarizine and flunarizine induce extrapyramidal reactions which seem to be mostly reversible [7, 24, 31]. This reversibility will be consistent with a dopaminergic blocking action and with the calcium antagonism [24]. But it will also be consistent with a deleterious antiperoxidant effect. As a matter of fact, activation of cells may require lipid peroxidation to generate the very active products of arachidonic acid. Cinnarizine has exerted its inhibitory effect on lipid peroxide formation in intact cells with its very minute amount, therefore, it seems reasonable to speculate the drug also decreases lipid peroxides very effectively *in vivo*. The decrease in lipid peroxides, moderate amounts of which are required to maintain normal and physiological cell activation, may induce the reduction in cell activation in substancia nigra.

Curiously, cinnarizine and manganese are both related to the induction of Parkinsonic syndromes and some of us have recently found manganese to be a potent inhibitor of copper-induced lipid peroxidation in both plasma and red-cells [32]. So, it is tempting to speculate that the dopaminergic neurotransmission system and its neurons may be affected by an antioxidant action. It is worthwhile to remember that Dormandy [33] has recently written "In Praise of Peroxidation" . . .

Acknowledgements—This work was supported in part by a grant from INIC. We would like to thank Mrs Maria do Céu Menezes for skilful technical assistance and Mrs Maria Celeste Fernandes for typing this manuscript.

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