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Antioxidant Action of the Antiarrhythmic Drug Mexiletine in Brain Membranes

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ABSTRACT—Mexiletine is a class Ib antiarrhythmic drug used in the treatment of ventricular arrhythmias. The Na^+ channel blocker mexiletine inhibits calcium influx in cells via decreasing reverse operation of the Na^+-Ca^{2+} exchanger. Thus this drug is shown to protect the CNS white matter against anoxic/ischemic injury. The aim of our study was to investigate if this drug could act as an antioxidant drug as well. The antioxidant action of this drug was studied under different oxidant conditions in vitro, and thiobarbituric acid−reactive substances were measured to follow lipid peroxidation. Mexiletine inhibited iron-ascorbate-H_2O_2−induced lipid peroxidation in brain membranes, liver microsomes and phospholipid liposomes, being most effective in brain membranes. The inhibition was dose- and time-dependent. Mexiletine also inhibited copper-ascorbate-H_2O_2−induced lipid peroxidation but to a lesser extent. It is concluded that mexiletine has a dual effect toward oxidative injury in brain, both by inhibiting Na^+-Ca^{2+} exchanger-dependent Ca^{2+} influx and by acting as an inhibitor of lipid peroxidation. However, as this drug is effective at millimolar concentrations, it should be considered less active than natural antioxidants that are effective at micromolar concentrations.

Keywords: Mexiletine, Antioxidant, Oxidative injury, Lipid peroxidation, Brain membrane

Mexiletine is a class Ib antiarrhythmic drug that acts by inhibiting cardiac Na^+ channels. Chronic administration of mexiletine results in the upregulation of cardiac Na^+ channel expression as several other class Ib drugs do (1−3). Treatment with mexiletine increases both the levels of mRNA encoding Na^+ channel α subunits and the number of Na^+ channels per cell. Na^+ channels are also modulated by polyunsaturated fatty acids (PUFA) (4). n-3 PUFA has been shown to inhibit Na^+ currents in a dose-, time- and voltage-dependent manner. In a recent study, Kang et al. showed that n-3 PUFA, unlike other class Ib antiarrhythmic drugs, does not upregulate the cardiac Na^+ channels, but does reduce the mexiletine-induced increase of Na^+ channel expression (1).

The stimulatory effect of increased cytoplasmic Ca^{2+} in the production of reactive oxygen species is well documented (5). Using the rat optic nerve as an in vitro model of white matter anoxia, Stys et al. showed that Ca^{2+} influx into axons played a key role in mediating irreversible injury (6) and the majority of the deleterious Ca^{2+} influx occurred via reverse operation of the Na^+-Ca^{2+} exchanger. Such an Na^+-Ca^{2+} exchanger-dependent Ca^{2+} import is confirmed by direct measurements (7, 8). Na^+ channel blockers such as tetrodoxin, local anesthetics, antiarrhythmics and certain anticonvulsants have been shown to be protective against anoxic injury in vitro. Stys and Lesiuk (9) showed that mexiletine was able to protect optic nerves from ischemic injury after systemic administration. They hypothesized that by blocking Na^+ channels, mexiletine inhibited Na^+-Ca^{2+} exchanger-dependent Ca^{2+} overload and decreased Ca^{2+}-dependent structural injury. (9) The effect of mexiletine was also studied in rat hearts subjected to global ischemia and reperfusion (10). Treatment of the perfused heart tissue with mexiletine has been shown to enhance post-ischemic contractile recovery and supress the changes in tissue Na^+, K^+, Ca^{2+} and Mg^{2+} contents. It has been suggested that the mechanism underlying the protective actions of mexiletine was the prevention of Na^+ overload and accompanying Ca^{2+} overload in cardiac cells (10).

In this study, our aim was to search for a direct antioxidant action of mexiletine. To eliminate the protective actions of mexiletine via Na^+ and Ca^{2+}-dependent mechanisms, we used tissue homogenate and lipid sub-

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strates instead of intact cells or tissues. We tested the direct antioxidant action of mexiletine on the peroxidation of different lipid substrates subjected to oxidant stress.

MATERIALS AND METHODS

Chemicals

Thiobarbituric acid (TBA), ascorbic acid (Asc), hydrogen peroxide (H_2O_2), phosphatidyl choline and phosphatidyl serine were from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA), ferrous sulfate, calcium chloride and copper chloride were from Merck (Germany). Mexiletine (1-(2,6-dimethylphenoxy)-2-propanamine) was from Boehringer (Mannheim, Germany).

Preparation of lipid substrates for peroxidation

Brain homogenate, liver microsomes and phospholipid liposomes were used to investigate the antioxidant actions of mexiletine. Only one male, 3-month-old Wistar rat of approx. 250 g was sacrificed for tissue preparations. The whole rat brain (approx. 3 g) was homogenized in 10 vol. of cold phosphate buffer (50 mM, pH 7.4) on ice using a dounce homogenizer. The resulting 10% (w/v) brain homogenate was used in lipid peroxidation studies.

The rat liver microsomes were prepared with calcium as described by Schenkman and Cinti (11). These microsomes were kept on ice-cold water and used for lipid peroxidation on the same day of preparation.

Phospholipids (phosphotidyl choline and phosphatidyl serine) were separately suspended in phosphate-buffered saline (PBS) at 2.5 mg/ml and then purged with nitrogen. These suspensions were vigorously shaken in stoppered glass bottles for 5 min to prepare liposomal suspensions. Resulting liposomes were stored at 4℃ for 1 h before use.

Determination of lipid peroxidation

The method of Ames et al. (12) was used to measure lipid peroxidation. Formation of thiobarbituric acid-reacting substances (TBARS) was used as a measure of lipid peroxidation. Peroxidation of lipid substrates was induced with ferrous iron (0.1 mM), copper (0.05 mM), Asc (1 mM) and H_2O_2 (1 mM) unless otherwise indicated. Peroxidizable lipids were incubated for 60 min under oxidant conditions as indicated in the legends to the Tables and Figs. The volume of the total reaction mixture was 1 ml for each assay. At the end of incubation, 0.5 ml of TCA (20%) was added to 1 ml of incubation mixture to stop the reactions. Samples were centrifuged at $1500 \times g$ for 10 min and 0.5 ml of supernatant was mixed with 0.5 ml of TBA (0.67%). Tubes were placed into boiling water for 20 min. After cooling the tubes, the absorbance of the samples was measured at 532 nm.

Determination of the antioxidant action of mexiletine

Iron (or copper)-Asc-H_2O_2-induced lipid peroxidation of lipid substrates was studied in the presence and absence of mexiletine. Mexiletine was added into the reaction mixture at the indicated concentrations just before the addition of lipid substrates. After 60 min of incubation under different conditions, the peroxidation reaction was terminated by the addition of TCA, and TBARS were determined as described above. The antioxidant activity of mexiletine is either shown on the Figs. or is expressed as percent inhibition of lipid peroxidation in the Tables.

All experiments were performed in triplicate, results are expressed as the mean or mean ± S.D. of experimental data. The difference between groups (with and without mexiletine) was analyzed by the two-tailed *t*-test and $P < 0.05$ was considered significant.

RESULTS

Mexiletine inhibited lipid peroxidation in rat brain homogenate. The time course of iron-Asc-H_2O_2-induced lipid peroxidation in brain homogenate is shown in Fig. 1. Lipid peroxidation was followed for 1 h and the inhibitory effect of mexiletine was studied at two different

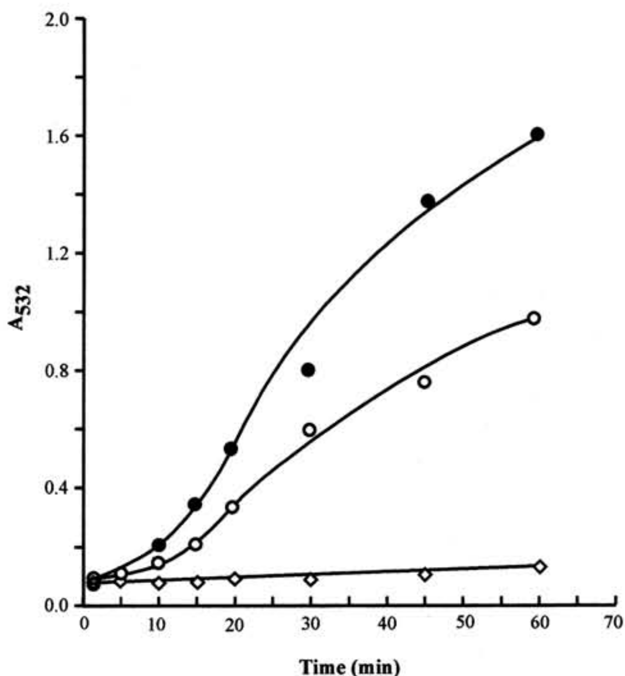

Fig. 1. The time course of iron-Asc-H_2O_2-induced lipid peroxidation in brain homogenate. Aliquots (1 ml) were drawn from the reaction mixture at the indicated time and pipetted onto TCA to stop the peroxidation reactions. ●, no mexiletine; ○, in the presence of 1.4 mM mexiletine; and ◇, in the presence of 14 mM mexiletine.

drug concentrations (1.4 and 14 mM). Mexiletine at 14 mM concentration (2.5 mg/ml assay mixture) completely inhibited lipid peroxidation.

The iron-Asc-H_2O_2 system is very effective for creating oxidant conditions (13). Under such conditions, the antioxidant effect of mexiletine is dependent on the drug concentration used. At 1 mg/assay (5.6 mM) concentration, mexiletine was more than 90% effective in protecting brain lipids against peroxidation (Fig. 2). The IC_{50} was calculated to be 1.9 mM.

The inhibition of lipid peroxidation by mexiletine was also studied using rat liver microsomes. Microsomal lipid peroxidation was studied using different oxidant conditions. Highest peroxidation was measured in the presence of iron, Asc and H_2O_2 (Table 1). Copper was less effective in stimulating microsomal lipid peroxidation. Under all the conditions studied, mexiletine inhibited the peroxidation of microsomal lipids (Table 1). Up to 84% inhibition of microsomal lipid peroxidation was observed at 1 mg/ml mexiletine concentration.

Mexiletine inhibited iron-Asc-H_2O_2-induced lipid peroxidation not only in biological membranes (as represented by brain homogenate and liver microsomes), but it also inhibited lipid peroxidation in phospholipid liposomes. Peroxidation of phosphatidyl choline liposomes induced by iron-Asc-H_2O_2 was inhibited by mexiletine in a dose-dependent manner (Fig. 3). However,

Table 1. Effect of mexiletine on lipid peroxidation in microsomes under different oxidant conditions

	(−) Mexiletine	(+) Mexiletine	% inhibition
No stimulation	0.096±0.005	0.111±0.01	—
Iron-Asc	0.472±0.019	0.075±0.006*	84
Iron-Asc-H_2O_2	0.501±0.009	0.087±0.005*	83
Copper-Asc	0.136±0.005	0.094±0.005*	30
Copper-Asc-H_2O_2	0.231±0.009	0.163±0.012*	29

Mexiletine concentration is 5.6 mM in each assay. Results are expressed as the mean±S.D., and the difference between groups (with and without mexiletine) was analyzed by the two-tailed *t*-test. *$P<0.05$.

when compared with brain homogenate (Fig. 2), the inhibition of liposome peroxidation was less pronounced (53% versus 95% inhibition in brain homogenate). Peroxidation of phosphatidyl serine lipsomes was studied under different conditions using iron and copper (Table 2). Maximum stimulation of phosphatidyl serine liposomes and highest inhibition by mexiletine occurred when peroxidation was induced with iron-Asc-H_2O_2 (Table 2). As in the case of liver microsomes, copper was a weaker stimulator of lipid peroxidation in phosphatidyl serine liposomes, and the inhibition level was even lower than the iron-induced peroxidation.

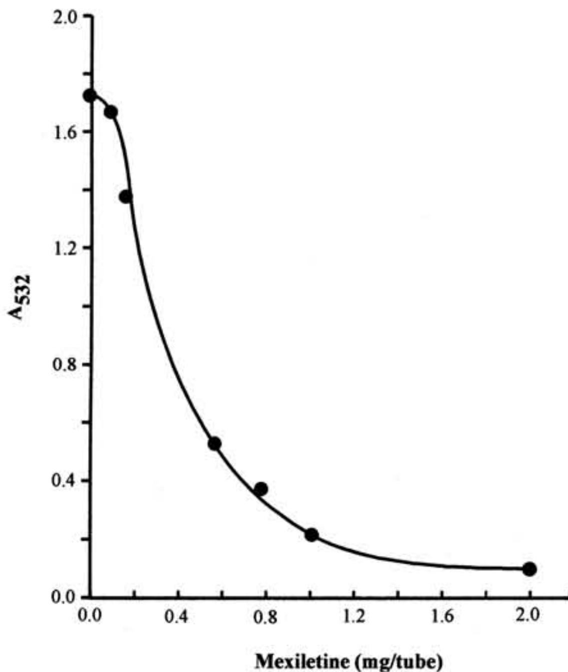

Fig. 2. Dose-dependent antioxidant action of mexiletine on lipid peroxidation in brain homogenates. Peroxidation was induced with iron-Asc and H_2O_2. After 60 min of incubation at room temperature, TBA reactivity was measured at 532 nm.

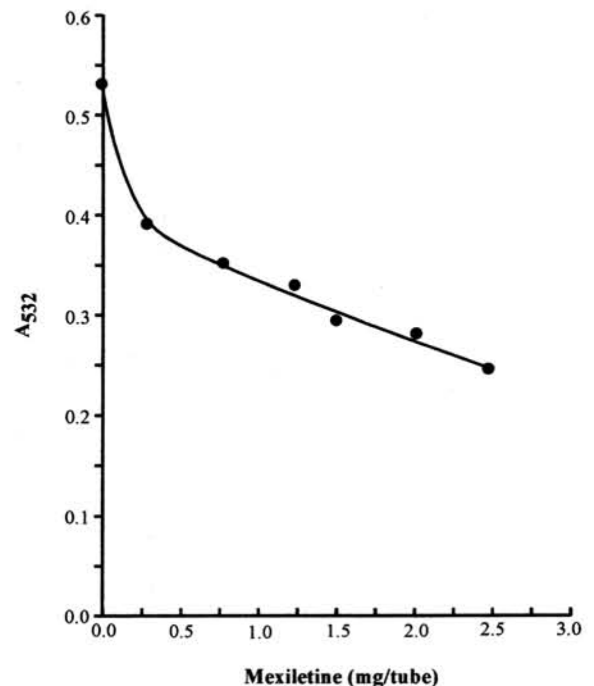

Fig. 3. Inhibition of iron-Asc-H_2O_2-induced lipid peroxidation of phosphatidyl choline liposomes by mexiletine. Two milligrams of phospholipid is used in each determination.

Table 2. Effect of mexiletine on lipid peroxidation in phosphatidyl serine liposomes under different oxidant conditions

	(−) Mexiletine	(+) Mexiletine	% inhibition
No stimulation	0.052±0.003	0.060±0.007	—
Iron-Asc-H_2O_2	0.523±0.011	0.201±0.015*	67
Asc-H_2O_2	0.218±0.016	0.142±0.01*	34
Iron-H_2O_2	0.184±0.004	0.151±0.011*	18
Copper-Asc-H_2O_2	0.229±0.006	0.118±0.007*	48
Copper-H_2O_2	0.080±0.007	0.064±0.003*	20

Two milligrams of phosphatidyl serine is used for each determination. Mexiletine concentration is 5.6 mM in each assay. Results are expressed as the mean±S.D., and the difference between groups (with and without mexiletine) was analyzed by the two-tailed *t*-test. *$P<0.05$.

DISCUSSION

As a Na^+ channel blocking agent, mexiletine is used as an antiarrhythmic drug. However, since chronic treatment with this drug results in the upregulation of Na^+ channels, treatment with this drug may itself cause arrhythmias as a consequence of therapy (1, 3). Therefore, careful reevaluation of the use of such drugs seems to be essential.

The Na^+ channel blockers are known to be highly protective against white matter anoxic injury (8, 14−16). This protection is explained by the fact that the blockage of Na^+ channels results in the inhibition of Na^+-Ca^{2+} exchanger-dependent Ca^{2+} overload. Recently, protective effect of mexiletine against white matter ischemia has been reported (2). In that study, it was concluded that the protonated form of mexiletine blocks the Na^+ channels and interrupts the anoxic cascade (2).

The aim of our present study was to investigate if mexiletine has any antioxidant action. For this purpose, we tested the effect of mexiletine toward lipid peroxidation in vitro, using the iron-Asc-H_2O_2 system to generate oxidant conditions. The most reactive oxygen species, hydroxyl radical (·OH), is produced from H_2O_2 in the presence of ferrous iron (Fenton reaction). Iron is oxidized to the ferric form in this process. In the presence of a reducing agent, such as superoxide or ascorbate, ferrous iron is regenerated from its oxidized form (13, 17). Hence, in the presence of a suitable reducer, a catalytic amount of iron can efficiently catalyze the formation of ·OH from H_2O_2 (13, 17).

Using iron-Asc-H_2O_2, we found that mexiletine at millimolar concentrations is a potent inhibitor of lipid peroxidation in vitro. The iron-Asc-H_2O_2 system is very effective for generating oxidant stress (Fig. 1). Up to 95% (for brain homogenate) and 83% (for the microsomes) protection against lipid peroxidation was observed at 5.6 mM mexiletine under the most oxidizing conditions (Fig. 2, Table 1). Mexiletine was less effective in protecting phospholipid liposomes from peroxidation (Fig. 3, Table 2). This suggests that mexiletine is a more effective antioxidant when interacted with biological membranes. Natural antioxidants are usually effective at micromolar concentrations (18). When compared with such antioxidants, it is clear that mexiletine is less active in inhibiting lipid peroxidation. However, we can still claim that in addition to inhibiting Ca^{2+} influx, mexiletine inhibits lipid peroxidation as does an antioxidant. Hence, mexiletine has a dual effect toward oxidative injury: both by inhibiting Ca^{2+} influx and acting as an inhibitor of lipid peroxidation.

By blocking Na^+ channels, mexiletine inhibits Na^+-dependent Ca^{2+} overload in the cells. The protection against anoxic/ischemic injury by mexiletine is explained by the inhibition of Na^+-Ca^{2+} exchanger with this drug (9). This study shows that in addition to the prevention of Ca^{2+} influx, mexiletine can function as an antioxidant in protecting cells against oxidant stress. Mexiletine is a highly hydrophobic compound containing only one polar group (-NH_2 group). One can assume that the methyl-substituted aromatic ring is incorporated into lipid bilayer with the amino group exposed to the aqueous phase. In our assay system, ·OH is expected to be generated in aqueous medium by iron-ascorbate-H_2O_2. Some of the ·OH can initiate the peroxidation of lipids. We hypothesize that mexiletine works via acting as an inhibitor of propagation of lipid peroxidation, probably similar to the action of α-tocopherol (an important chain-breaking antioxidant in biological membranes). Further studies are essential to identify the molecular mechanism of the antioxidant action of mexiletine.

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