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The antioxidant activity of caroverine

Natalia Udilova[a], Andrey V. Kozlov[a], Werner Bieberschulte[b], Klemens Frei[c], Klaus Ehrenberger[c], Hans Nohl[a,*]

[a]*Institute of Pharmacology and Toxicology, University of Veterinary Medicine Vienna, Veterinaerplatz 1, A-1210 Vienna, Austria*
[b]*PHAFAG AG, FL-9486 Schaanwald, Liechtenstein*
[c]*Department of Otorhinolaryngology, General Hospital of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria*

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Abstract

Caroverine, 1-(2-diethylaminoethyl)-3-(*p*-methoxy benzyl)-1,2-dihydro-2-quinoxalin-2-on-hydrochloride, is a class B calcium-channel-blocker and antiglutamatergic agent with significant effects on the brain function. Caroverine exhibits competitive AMPA antagonism, and at higher concentrations, noncompetitive NMDA antagonism. In clinical practice caroverine is used as a spasmolytic and otoneuroprotective agent. Since reactive oxygen species are supposed to be involved in the pathogenesis of inner ear diseases in which caroverine shows beneficial effects, the present study aimed to investigate the antioxidant properties of caroverine. Lipid peroxidation of liposomal membranes was suppressed in the presence of caroverine. In order to understand the mechanism of this antioxidant action of caroverine, we determined the rate constants both for a possible reaction with superoxide ($O_2^{\bullet -}$) radicals from xanthine/xanthine oxidase and for a possible reaction with hydroxyl ($^{\bullet}OH$) radicals in Fenton system. Using a defined chemical reaction model $O_2^{\bullet -}$ scavenging was found to occur at a rather low rate constant only (3×10^2 M^{-1} s^{-1}). Thus, a reaction of caroverine with $O_2^{\bullet -}$ radicals is of marginal significance. In contrast, the reaction of caroverine with $^{\bullet}OH$ radicals occurs at an extremely high rate constant ($k = 1.9 \times 10^{10}$ M^{-1} s^{-1}). The strong antioxidant activity of caroverine is therefore based both on the partial prevention and highly active scavenging of hydroxyl radicals.

Keywords: Caroverine; Antioxidant; Lipid peroxidation; Iron; ESR; Spasmolytic drug

1. Introduction

Caroverine, 1-(2-diethylaminoethyl)-3-(*p*-methoxy benzyl)-1,2-dihydro-2-quinoxalin-2-on-hydrochloride (Scheme 1), is a class B calcium-channel-blocker [1] and antiglutamatergic agent with significant effects on the brain function [2–6]. Caroverine exhibits competitive AMPA antagonism, and at higher concentrations, noncompetitive NMDA antagonism.

Caroverine is chemically derived from isoquinoline, the basic structure of papaverin. Like papaverin, caroverine has spasmolytic properties [7] which may be linked to its Ca^{2+}-channel blocking activities. In combination with antagonistic effects on glutamate and aspartate receptors these multifunctional activities may be responsible for a variety of beneficial results in the therapy of tinnitus, sudden hearing loss, speech discrimination disorders and other neurotoxic effects, such as ischemia/reperfusion, hypoglycemia, anoxia, hypoxia, shock and dementia [8–12]. In many of these pathophysiological events reactive oxygen species (ROS) play an important role. The most susceptible target of ROS are biomembranes where lipid peroxidation (LPO) occurs. LPO affects the structure and function of biomembranes and LPO-derived metabolites exert cytotoxic and genotoxic effects [13–16].

The physiological control of the formation and the pathogenetic threat of ROS is performed by enzymatic and nonenzymatic antioxidants. Depending on the duration of oxidative stress causing LPO, the native antioxidant system can be impaired or exhausted. Oxidative stress

* Corresponding author. Tel.: +43-1-25077-4400; fax: +43-1-25077-4490.
E-mail address: hans.nohl@vu-wien.ac.at (H. Nohl).

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazone-propionic acid; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; ESR, electron spin resonance; NMDA, *N*-methyl-D-aspartate; LPO, lipid peroxidation; ROS, reactive oxygen species; SOD, superoxide dismutase; $^{\bullet}OH$, hydroxyl radical; $O_2^{\bullet -}$, superoxide anion radical.

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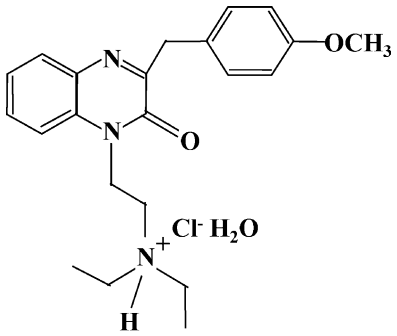

$C_{22}H_{28}N_3O_2Cl \cdot H_2O$

Scheme 1. Chemical structure of caroverine.

associated with the impairment of antioxidant defense is involved in a great variety of metabolic disorders such as diabetes mellitus, neuromuscular diseases, cardiovascular diseases, cancer and the biological process of aging [17–24]. Thus, a still increasing spectrum of diseases as well as aging has become subject of antioxidant supplementation. Since among all reactive oxygen species the hydroxyl radical (•OH) is by far the most potent and therefore the most dangerous oxygen metabolite, elimination of this radical is one of the major aims of antioxidant administration. Although a variety of •OH scavengers are known, their application is limited by (1) the requirement of unphysiologically high scavenger concentrations which depend on the rate constant, (2) toxic side effects including initiation of further radical chain reactions, or (3) the instability of the compound in biological systems.

Caroverine is introduced since a couple of years in medical treatment of neurological diseases exhibits beneficial effects which cannot be explained exclusively on the basis of Ca^{2+}-channel blockage or antagonism of AMPA and NMDA receptors. The present study was made in order to test the potential antioxidant activity of caroverine.

2. Materials and methods

2.1. Chemicals

Caroverinehydrochloride monohydrate (caroverine), 1-(2-diethylaminoethyl)-3-(*p*-methoxy benzyl)-1,2-dihydro-2-quinoxalin-2-on-hydrochloride, was obtained from PHAFAG AG. Although caroverine dissolves poorly in water, it was possible to obtain stock solutions of 50 mM by vigorous mixing using a vortex mixer.

Hydrogen peroxide, thiobarbituric acid, diethylenetriaminepentaacetic acid, Vitamin E, $FeSO_4 \cdot 7H_2O$, and KH_2PO_4 and solvents were obtained from Merck. DMPO, xanthine, xanthine oxidase and cytochrome *c* were obtained from Sigma. All the chemicals were of analytical grade purity.

2.2. Liposomes preparation

Liposomes were prepared from soybean phosphatidylcholine (Sigma). A chloroform solution of phosphatidylcholine was dried under vacuum and the lipid film was dissolved in 200 mM phosphate buffer (pH 7.4) so that final concentration of lipid was 1 mg/mL. In order to produce multilamellar liposomes with low lipid hydroperoxide content, lipid emulsion was rapidly frozen in liquid nitrogen and subsequently thawed, the procedure being repeated five times. When indicated, Vitamin E (1/100 mol/mol lipid) was added to the phosphatidylcholine chloroform solution prior to evaporation.

2.3. Initiation of lipid peroxidation by Fenton reagents

In order to start lipid peroxidation, 1 mM hydrogen peroxide was added to phosphatidylcholine liposomes containing Vitamin E and the reaction was started by 1 mM iron(II). After 2 hr incubation at room temperature the reaction was stopped by the addition of butylated hydroxytoluene (final concentration 0.5 mg/mL). Caroverine or its hydrochloride was added as 50 mM solution in ethanol or water, respectively prior to H_2O_2 and iron(II) reagents.

2.4. Vitamin E measurements

Vitamin E was extracted from liposomes with hexane and measured by HPLC with fluorescence/UV-detection as described earlier [25].

2.5. Determination of hydroxyl radical scavenging activity

DMPO was purified by activated carbon under nitrogen. Concentrations of DMPO and H_2O_2 were determined from their absorption spectra at 230 nm (7700 M^{-1} cm^{-1}) and 240 nm (39 M^{-1} cm^{-1}), respectively. Rate constants for the reaction of caroverine with •OH radicals were determined in air-saturated pH 7.0 phosphate buffer (10 mM) at room temperature. The reaction was initiated by the addition of a small aliquot of Fe^{2+} solution (100 mM $FeSO_4$ in 10 mM HCl) to a buffer containing caroverine, H_2O_2, and DMPO. The final volume of every sample was 0.5 mL. The final concentrations of the reagents were: 1 mM (or 0.1 mM) $FeSO_4$; 1 mM (or 0.1 mM) H_2O_2, 1 mM DMPO; 1–600 μM caroverine. The sample was transferred quickly to a flat quartz aqueous ESR cell and measurements were started immediately. ESR spectra were measured using a EMX Bruker ESR spectrometer operating at 9.7 GHz.

To determine the reaction rate of caroverine with •OH radicals the kinetic model reported earlier for the reaction of ethanol with •OH [26,27] was adopted. According to the

kinetic model, the concentration of hydroxy radicals [•OH] can be written as follows:

$$\frac{d[^{\bullet}OH]}{dt} = k[Fe^{2+}][H_2O_2] - k_d[^{\bullet}OH][DMPO] - k_c[^{\bullet}OH][caroverine] \quad (1)$$

where $[Fe^{2+}]$, $[H_2O_2]$, [DMPO], and [caroverine] represent the concentrations of iron(II), hydrogen peroxide, DMPO and caroverine, respectively, whereas k, k_d and k_c represent the respective reaction constants. Using the steady state approximation ($d[^{\bullet}OH]/dt = 0$) for [•OH]), Eq. (1) can be rewritten as:

$$k[Fe^{2+}][H_2O_2] - k_d[^{\bullet}OH][DMPO] - k_c[^{\bullet}OH][caroverine] = 0 \quad (1a)$$

The velocity of DMPO/•OH adduct formation is indicated as $V^{DMPO\bullet}$:

$$\frac{d[DMPO^{\bullet}]}{dt} = V^{DMPO\bullet} = k_d[^{\bullet}OH][DMPO] \quad (2)$$

From Eq. (2) the concentration of hydroxy radicals can be drawn as:

$$[^{\bullet}OH] = \frac{V^{DMPO\bullet}}{k_d[DMPO]} \quad (3)$$

The production velocity of hydroxy radicals is indicated as V^{OH}:

$$V^{OH} = k[Fe^{2+}][H_2O_2] \quad (4)$$

Substituting Eqs. (3) and (4) into Eq. (1a) we obtain:

$$V^{OH} - V^{DMPO\bullet} - \frac{V^{DMPO\bullet}k_c[caroverine]}{k_d[DMPO]} = 0 \quad (5)$$

Eq. (5) can be rewritten as follows:

$$\frac{V^{OH}}{V^{DMPO\bullet}} - 1 = \frac{k_c}{k_d[caroverine]/[DMPO]} \quad (6)$$

in which $V^{DMPO\bullet}$ and V^{OH} represent formation rates of DMPO/•OH adduct and •OH radicals, and k_d and k_c represent the rate constants for the reaction of •OH with DMPO and with caroverine, respectively. V^{OH} can be measured as DMPO/•OH formation rate in the absence of caroverine, since from Eq. (6) if [caroverine] = 0 then $V^{OH} = V^{DMPO\bullet}$.

A plot of $(V^{OH}/V^{DMPO\bullet} - 1)$ vs. ([caroverine]/[DMPO]) should give a straight line with zero as the intercept and k_c/k_d as the slope. Since the reaction rate constant of DMPO with hydroxyl radicals k_d is known, the rate constant of caroverine with hydroxyl radicals k_c can be calculated from the slope.

Thus, we determined each time V^{OH} in a parallel experiment in the absence of caroverine, using formula 6 for determination of the rate constant.

It has been previously proven that rate constants obtained using rates of the reaction, agree very well with those calculated by using the corresponding amplitudes of the respective ESR-signals of DMPO/•OH adducts measured at different concentrations of •OH scavengers [28].

2.6. Determination of superoxide scavenging activity

The rate constant for the reaction of caroverine with the superoxide radical was determined by competitive kinetics using cytochrome *c* as a competitive reagent. Rate constants for the reaction of caroverine with $O_2^{\bullet -}$ radicals were determined in air-saturated pH 7.8 phosphate buffer (50 mM), containing 0.1 mM diethylenetriaminepentaacetic acid at room temperature. The reaction was initiated by the addition of a small aliquot of xanthine to a buffer containing caroverine, cytochrome *c*, and xanthine oxidase. The final volume of every sample was 3 mL. The final concentrations of the reagents were: 0.1 mM xanthine; 0.01 U/probe xanthine oxidase; 10 μM cytochrome *c*. The optical spectra were recorded using Hitachi U-3300 spectrophotometer. The concentration of reduced cytochrome *c* was estimated by measuring absorbance at 550 nm.

To determine rate constants for scavenging of $O_2^{\bullet -}$ by caroverine we used the same kinetic model as described earlier for •OH.

2.7. Statistics

If not indicated otherwise, all experiments were repeated at least five times. Statistic parameters were calculated using ANOVA (Exel 5.0 software, Microsoft Inc.).

3. Results

Lipid peroxidation (LPO) has two important consequences, namely accumulation of lipid degradation products and depletion of antioxidants. Depletion of antioxidants indicates that LPO runs out of control leading *in vivo* to cell and tissue damage. Frequently used model systems for studying LPO *in vitro* is the exposure of lipid membranes to a mixture of ferrous iron and H_2O_2 (Fenton system). The latter system yields •OH-radicals which are the most potent oxidants existing in aerobic living systems.

Fig. 1 presents the effect of caroverine on the initiation of LPO in liposomal vesicles. Liposomes were preloaded with Vitamin E allowing to follow LPO by the extend of Vitamin E depletion. LPO was initiated by exposing liposomes to a Fenton-type system. The onset of Vitamin E depletion started already 15 min after the initiation of LPO (not shown). The addition of 62 μM caroverine was sufficient to totally suppress Vitamin E consumption.

The partition coefficient of caroverine was determined in the octanol/water mixture. The value of 1.8 reveals that radicals can be scavenged both in the polar and lipid phase of membranes.

To get insight into the site of caroverine interaction in the cascade of Fenton-induced LPO, we investigated

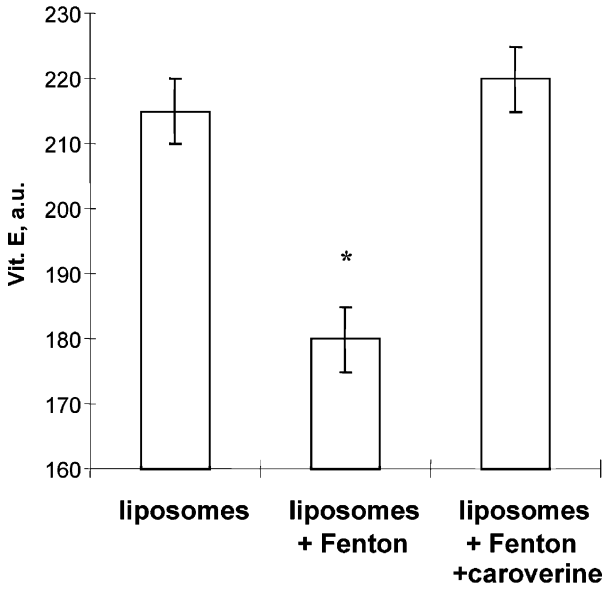

Fig. 1. Inhibition of LPO induced by Fenton reagent in the presence of caroverine. About 400 μL phosphatidylcholine liposomes (1 mg/mL) loaded with Vitamin E (1/10 mol/mol lipid) were incubated for 2 hr at room temperature without any additions or with Fenton reagents (1 mM H_2O_2 + 1 mM Fe^{2+}) in presence and absence of 62 μM caroverine. The reaction was stopped by addition of 10 μL butylated hydroxytoluene (20 mg/mL) and Vitamin E content was analyzed as described in Section 2. Values are means ± SEM (N = 3). Key: (*) $P < 0.05$ vs. control (Student's t-test).

whether or not caroverine scavenges •OH radicals. For this purpose we generated •OH-radicals from a Fenton system. The radicals formed were scavenged and quantified by the spin trap DMPO giving rise to a characteristic 1:2:2:1 ESR quartet spectrum (Fig. 2A) ($a_N = 14.9$ G, $a_H = 14.8$ G). Caroverine was found to decrease the quartet ESR-signal in a concentration dependent manner (Fig. 2B). To examine whether caroverine attenuates the ESR-signal by a direct interaction with DMPO/•OH adduct, we first generated the spin adduct in the absence of caroverine. In a second experiment caroverine was added after the DMPO/•OH adduct was formed. The amplitude of the corresponding ESR-signal was essentially the same, excluding a direct effect of caroverine on the DMPO-spin adduct (data not shown). We therefore focused our interest on a direct •OH-scavenging activity of caroverine.

The most valid indicator for an evaluation of radical scavenging potency is the rate constant by which the antioxidant reacts with the radical. The respective rate constant for caroverine was calculated from the dependence of the DMPO/•OH signal amplitude on caroverine concentration using a kinetic model system described in the Section 2. •OH generation rates were not changed (Fig. 2B). The rate constant determined from this calculation procedure was found to be 1.5×10^{10} M^{-1} s^{-1} if 0.1 mM

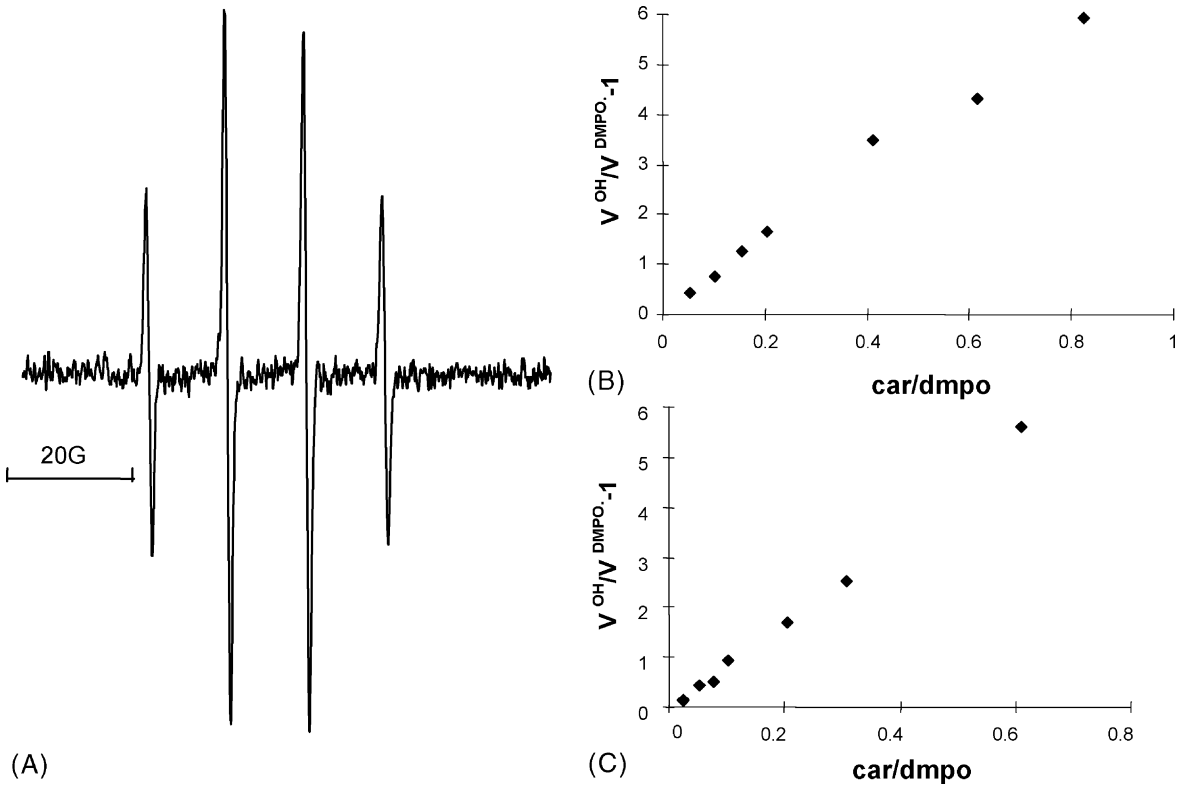

Fig. 2. Determination of the rate constant of the reaction between caroverine and •OH radicals. (A) ESR spectrum recorded 1 min after mixing 1 mM $FeSO_4 \cdot 7H_2O$, 1 mM H_2O_2, and 1 mM DMPO in phosphate buffer solution. Both (B) and (C) show competition between DMPO and caroverine for •OH radicals in 10 mM phosphate buffer (pH 7) containing 1 mM DMPO. The •OH radicals were produced by the reaction of 1 mM $FeSO_4 \cdot 7H_2O$ with 1 mM H_2O_2 (B) or 0.1 mM $FeSO_4 \cdot 7H_2O$ with 0.1 mM H_2O_2 (C) in the presence of 1 mM DMPO. The data were plotted according to kinetic model described in Section 2.

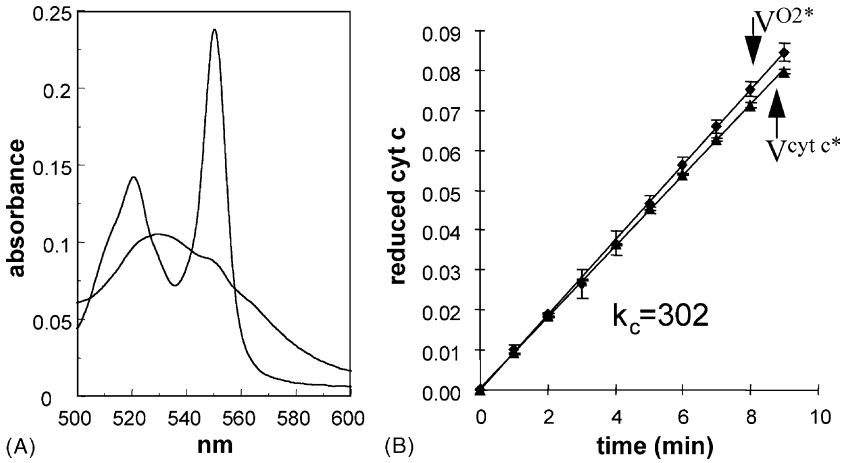

Fig. 3. Superoxide scavenging capacity of caroverine. (A) Optical spectra of oxidized and reduced forms of cytochrome *c* used as a competitive scavenger of superoxide radicals. (B) Kinetics of cytochrome *c* reduction in the presence (▲) and in the absence (◆) of caroverine. The reaction was initiated by the addition of xanthine (0.1 mM final concentration) to a buffer containing 763 μM caroverine, 10 μM cytochrome *c*, 0.01 U xanthine oxidase. The final volume of the sample was 3 mL. Values are means ± SEM (N = 5).

Fe^{2+} was used in Fenton reagent and 1.9×10^{10} M^{-1} s^{-1} if 1 mM iron was used in Fenton reagent. These rate constants are diffusion limited revealing an extremely high reactivity of caroverine with $^{\bullet}OH$ radicals. The small increase of the rate constant observed when ferrous iron concentration was elevated by a factor of 10 may be taken as an indicator that caroverine also reacts with ferrous iron.

In biological systems H_2O_2 which is the precursor of $^{\bullet}OH$ is provided by the dismutation of superoxide radicals ($O_2^{\bullet -}$). Thus, it was of interest also to test the ability of caroverine to scavenge $O_2^{\bullet -}$ radicals.

The rate constant for the reaction of caroverine with the $O_2^{\bullet -}$ was determined by competitive kinetics using cytochrome *c* as a competitive reagent. Cytochrome *c* was used for detection of superoxide. The production of $O_2^{\bullet -}$ (from xanthine/xanthine oxidase) in the presence of cytochrome *c* results in its SOD-sensitive reduction with characteristic changes in optical spectra (Fig. 3A).

The reduction of cytochrome *c* was not significantly inhibited even in the presence of high concentrations of caroverine (Fig. 3B). The rate constant of the reaction between caroverine and superoxide calculated from these experiments was 3×10^2 M^{-1} s^{-1}. This is much lower than the corresponding constant for SOD ($\approx 10^{10}$ M^{-1} s^{-1}) [29] and cytochrome *c* ($\approx 10^{5\ -1}$ s^{-1}) [30]. Thus, the superoxide scavenging capacity is not likely to explain the antioxidant activity of caroverine.

Apart from $O_2^{\bullet -}$ derived H_2O_2, iron plays a major role as catalyst in the establishment of oxidative stress. This holds both for our *in vitro* as well as for the *in vivo* formation of $^{\bullet}OH$ radicals. Removal of free iron therefore downregulates oxidative stress. Although, caroverine was

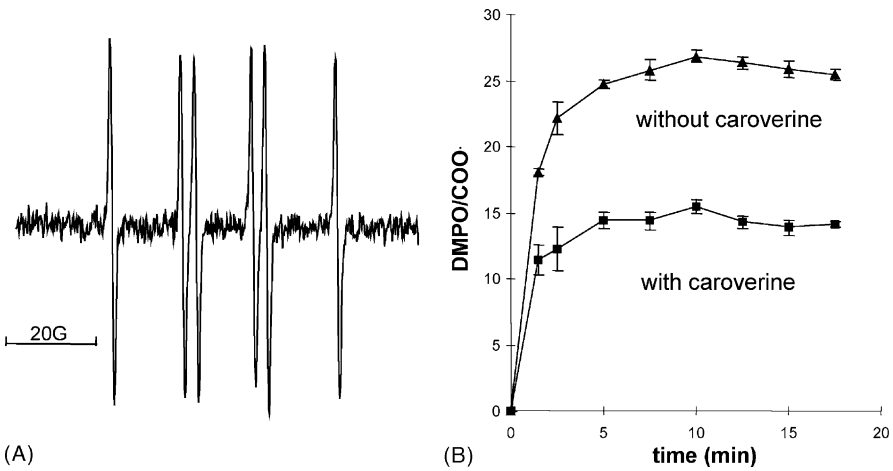

Fig. 4. Effect of caroverine on formate radical levels in Fenton system in the presence of formate. (A) ESR spectrum of DMPO/$COO^{\bullet -}$ adduct recorded 1 min after mixing 1 mM $FeSO_4 \cdot 7H_2O$, 1 mM H_2O_2, 1 mM DMPO, and 40 mM formate in phosphate buffer solution. (B) Inhibition of DMPO/$COO^{\bullet -}$ adducts formation in the presence of 200 μM of caroverine ($P < 0.001$, Student's *t*-test). Further increase of caroverine concentration did not result in a decrease of DMPO/$COO^{\bullet -}$ levels. Values are means ± SEM (N = 5).

shown to intensively intercept •OH-related DMPO-adduct formation, it is not clear whether this compound interferes into •OH-generation or whether it predominantly scavenges •OH radicals. We therefore designed another type of experiment in which iron derived •OH radicals were allowed to react with formate. Reaction of formate with •OH generates formate-derived carbon-centered $COO^{\bullet -}$ radicals with a reaction rate of $3.9 \times 10^9\ M^{-1}\ s^{-1}$. Formate-derived radicals will react with DMPO to produce another type of ESR spectra which is related to DMPO/$COO^{\bullet -}$ adduct. The reaction rate of DMPO with •OH is $3.4 \times 10^9\ M^{-1}\ s^{-1}$ [31]. Since both reaction rates are comparable, •OH radicals will predominately react with formate to generate $COO^{\bullet -}$ because formate was present in excess. In that case the major ESR-signal will be the DMPO/$COO^{\bullet -}$-derived sextet (Fig. 4A).

The measurements showed that reaction of Fe(II) with H_2O_2 in the presence of DMPO and 40 mM formate indeed generated DMPO/$COO^{\bullet -}$ as a major spin-adduct signal ($a_N = 15.6$ G; $a_H = 18.7$ G)(Fig. 4A). If caroverine were an •OH scavenger only, then the intensity of DMPO/$COO^{\bullet -}$ signal would not change in the presence of formate. However, our results clearly show, that the addition of caroverine affects the intensity of the DMPO/$COO^{\bullet -}$ adduct signal (Fig. 4B). Thus, it appears that caroverine is capable both of scavenging •OH radicals and intervening into the Fenton reaction. Since caroverine inhibits DMPO/$COO^{\bullet -}$ adduct formation by a factor of 2, one can conclude that at least 50% of the antioxidant activity of caroverine is due to •OH scavenging and the other half is likely due to inhibition of Fenton reaction.

4. Discussion

The present study provides experimental evidence that caroverine besides its various pharmacological activities has also strong antioxidant properties. This novel finding may be a rational to understand the beneficial effects in a variety of diseases in which oxidative stress was long known to contribute to the respective pathogenesis. Caroverine removes •OH radicals, the most dangerous oxygen metabolites formed in aerobic organisms. The rate constant for the reaction of caroverine with •OH radicals is diffusion controlled (mean value of $1.71 \pm 0.22 \times 10^{10}\ M^{-1}\ s^{-1}$) and exceeds by one order of magnitude the reaction velocities of hydroxyl radicals with most biomolecules. This also includes the native biological •OH scavengers such as mannitol, uric acid or bilirubin. Caroverine can therefore be considered to efficiently protect biomolecules from oxidative stress. The rate constant between caroverine and •OH radicals is close to that determined earlier for tryptophan [32] and several other indolic compounds [33].

The efficiency of a substance to scavenge radicals depends both on the respective rate constant and a concentration of the substance. In case of caroverine, a dose up to 160 mg twice a day (b.i.d.) i.v. is usually applied and well tolerated [8]. From this dose and from the relatively low octanol/water partition coefficient of caroverine, its maximal concentration in blood can be estimated. Taking into account the rate constant of the reaction between caroverine and hydroxyl radicals, the scavenging potency of caroverine *in vivo* can also be roughly estimated. It was found to be in the range of other natural hydroxyl radical scavengers present in blood such as uric acid, glucose and albumin indicating that contribution of caroverine to the whole radical scavenging activity of plasma may be of physiological significance.

The antioxidant activity of caroverine is not only restricted to the potent removal of •OH-radicals. Caroverine was also shown to interfere into •OH-radical generation. According to our results, the catalytic role of ferrous iron in the reductive homolytic cleavage of H_2O_2 seems to be impaired in the presence of caroverine.

Thus, an advantage of caroverine with respect to the natural scavengers mentioned above, is its potency not only to remove •OH radicals but also to inhibit the establishment of oxidative stress. Caroverine is successfully used in the treatment of tinnitus, sudden hearing loss and progressive hearing loss [8,12]. Reactive oxygen species and changes in intracochlear blood flow are supposed to be involved in hair cell damage during age-related hearing loss [34]. Glutamate acts as an excitory neurotransmitter at the inner hair cells and the afferent neuron by a binding to NMDA and AMPA receptors located on the postsynaptic afferent membrane [35]. Glutamatergic neurotoxicity is caused by overproduction of reactive oxygen species and following membrane damage by lipid peroxidation [36]. The demonstrated neuroprotective therapeutic effects of caroverine confirm the working hypothesis of glutamatergic otoneurotoxity causing symptoms as tinnitus, sudden hearing loss or progressing hearing loss. Additional evidences for the involvement of reactive oxygen species in the inner ear diseases come from the study which demonstrates a protective effect of antioxidant supplementation on age-related hearing loss in rats [37]. Thus, the antioxidant properties of caroverine may contribute to its beneficial effects in the treatment of inner ear diseases.

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