Volume 294, number 3, 264–266 FEBS 10489 December 1991

Pyridoindole stobadine is a potent scavenger of hydroxyl radicals

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Received 30 September 1991

Stobadine is a potent scavenger of $OH^{\bullet}$ radicals generated chemically in a free solution with k_2 higher than $10^{10} \cdot M^{-1} \cdot s^{-1}$ as determined by two independent methods, namely destruction of deoxyribose and oxidation of 2-keto-4-methiolbutyric acid (KMBA). The high efficacy of stobadine to prevent ethylene production from KMBA was observed also in enzymatic (xanthine-xanthine oxidase-driven Fenton) and membrane-bound (NADPH-dependent microsomal electron transfer) sources of $OH^{\bullet}$ radicals.

Pyridoindole; Stobadine; Hydroxyl radical scavenger; Deoxyribose assay; KMBA assay

1. INTRODUCTION

Stobadine, a novel drug with the pyridoindole structure (−)-*cis*-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1*H*-pyrido-[4,3-b]indole, was found to exhibit antiarrhythmic properties and proved efficient in treating acute ischemia following myocardial infarction [1]. The antioxidant properties of stobadine, demonstrated by its ability to prevent lipid peroxidation in ischemic-reperfused brain tissue [2], microsomal membrane of liver [3], and model phosphatidylcholine liposomes [4], were suggested to account for the mechanism of the cardioprotective action of the drug [5].

Since Ondrias et al. [4] showed the ability of stobadine to compete with DMPO in trapping $OH^{\bullet}$ radicals in the ESR system, we considered it worthwhile to study the interaction of this drug with $OH^{\bullet}$ in more detail. In the present work we used two chemical methods for detecting $OH^{\bullet}$ radicals, namely deoxyribose oxidation to thiobarbituric (TBA)-reactive products, and ethylene production from 2-keto-4-methiolbutyric acid (KMBA).

2. MATERIALS AND METHODS

2.1. *Reagents*

Stobadine dihydrochloride was synthesized at the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague. Deoxyribose, KMBA, xanthine, xanthine oxidase, $NADP^+$, glucose 6-phosphate, glucose 6-phosphate dehydrogenase were from Sigma. TBA was from Fluka. Other chemicals were obtained from local commercial sources and were of analytical grade quality.

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2.2. *Deoxyribose assay*

Degradation of deoxyribose by $OH^{\bullet}$ was measured as in [6]. Hydroxyl radicals were generated chemically by a mixture of Fe(III), EDTA, ascorbic acid and H_2O_2. The reaction mixtures contained the following reagents at the final concentrations: 20 mM phosphate buffer, pH 7.4, 100 μM EDTA, 20 μM $FeCl_3$, 2.8 mM deoxyribose, 1 mM H_2O_2 and 0.1 mM ascorbic acid. Tube contents (1.0 ml) were incubated at 37°C for 1 h, then TBA-reactive products were determined as in [7].

2.3. *KMBA assay*

The production of ethylene from KMBA was assayed by the headspace gas chromatography procedure as in [8]. The chemical model system used to generate $OH^{\bullet}$ consisted of 0.2 mM EDTA, 0.1 mM $Fe(NH_4)_2(SO)_4)_2$ and 1.7 mM ascorbic acid in 20 mM phosphate buffer, pH 7.4. Two enzymatic model systems were used to generate $OH^{\bullet}$ radicals: one was the xanthine-xanthine oxidase-driven Fenton reaction. The reaction mixture contained 20 mM phosphate buffer, pH 7.4, 20 μM EDTA, 5 μM $Fe(NH_4)_2(SO)_2$, 0.5 mM xanthine, 0.025 U of xanthine oxidase and 50 μM H_2O_2 in a final volume of 1.5 ml; the second was the membrane-bound NADPH-dependent microsomal electron transfer system. Hepatic microsomes from male Wistar rats were prepared as described elsewhere [3]. The reaction system consisted of 40 mM phosphate buffer, pH 7.4, 6 mM $MgCl_2$, 6 mM glucose 6-phosphate, 0.6 mM $NADP^+$, 1 U/ml glucose 6-phosphate dehydrogenase, 1 mM sodium azide, 25 μM $Fe(NH_4)_2(SO_4)_2$, 50 μM EDTA and about 1.5 mg/ml microsomal protein. The reaction mixtures were incubated at 27°C in the presence of various amounts of stobadine and 1 mM KMBA for 60 min.

3. RESULTS

3.1. *Deoxyribose assay*

Deoxyribose degradation was efficiently diminished by stobadine. Fig. 1 shows that in the concentration range 0.1–2 mM the inhibition by stobadine exhibits simple competition kinetics (linear correlation >0.98 for each experiment). From the slopes of the experimental lines approximate values of the second-order rate constant for the reaction between stobadine and $OH^{\bullet}$ were calculated. In a series of 6 experiments the average value obtained was (+/− SEM) 1.59 +/− 0.11 × $10^{10} \cdot M^{-1} \cdot s^{-1}$.

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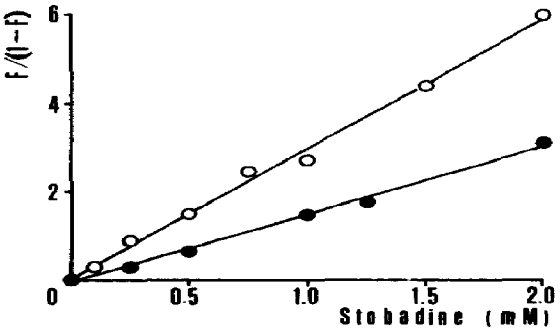

Fig. 1. Inhibition of OH•-dependent deoxyribose degradation (○) and KMBA oxidation (●) by stobadine in pure chemical systems producing hydroxyl radicals. For experimental conditions see Materials and Methods. The rate constant k_S was determined [9] from the slope of the lines $F/1-F = k_S/k_D \cdot [D] \times [S]$, where F is the percentage inhibition value at a particular concentration of the scavenger [S] and at the fixed concentration of the detection molecule [D]. The rate constants, k_D, used in the calculations (expressed as $\cdot M^{-1} \cdot s^{-1}$) were 1.9×10^{10} for deoxyribose [10] and 7.8×10^{10} for KMBA [9]. The kinetic plots are results from two representative experiments.

3.2. *KMBA assay*

In order to confirm the OH•-scavenging ability of stobadine, a completely different detection method based on the oxidation of KMBA [8] was used. Stobadine effectively prevented oxidation of KMBA, with the IC_{50} value shown in Table I. In the concentration range 0.25–2 mM simple competition plots were obtained (Fig. 1), with good linear correlation (>0.98) for each experiment, which gave the average (+/−SEM) value of $1.41 +/- 0.17 \times 10^{10} \cdot M^{-1} \cdot s^{-1}$ (n=6) for the second-order rate constant of stobadine.

The inhibition effect of stobadine on KMBA oxidation was studied in two other OH•-generating systems: an enzymatic system, i.e. the xanthine-xanthine oxidase-driven Fenton reaction, and a membrane-bound enzymatic system, i.e. NADPH-dependent microsomal electron transfer. In both systems stobadine suppressed ethylene formation with an efficacy comparable to that observed in the pure chemical system (Table I).

Table I

Stobadine inhibition of ethylene production from KMBA in different OH•-generating systems

System studied*	IC_{50} value (mM)
Ascorbate-Fe-EDTA	0.81 +/− 0.10 (6)
Xanthine-Xanthine oxidase-Fe-EDTA	0.93 +/− 0.14 (5)
Microsomes-NADPH-Fe-EDTA**	0.74 +/− 0.21 (5)

*For complete experimental conditions see Materials and Methods. Results are mean values +/− SEM with the number of experiments in parentheses.
**In the absence of NADPH-generating system no measurable amount of ethylene was produced.

Table II

Summary of experimental values of the second-order rate constants for the reaction of stobadine with OH• radicals

Method	Source of OH•	$k_S \times 10^{10}$ [$M^{-1} \cdot s^{-1}$]
KMBA assay	Ascorbate-Fe-EDTA	1.41 +/− 0.17 (6)
Deoxyribose assay	Ascorbate-Fe-EDTA-H_2O_2	1.59 +/− 0.11 (6)
DMPO spin trapping	Fe-ADP-H_2O_2	1.7*

*Calculated from the experimental data of Ondrias et al. [4]

4. DISCUSSION

Stobadine was found to be a powerful scavenger of OH• radicals. Its OH•-scavenging ability is characterized by a second-order rate constant higher than $1 \times 10^{10} \cdot M^{-1} \cdot s^{-1}$ in both deoxyribose and KMBA assays (see Table II).

Using ESR spectroscopy, Ondrias et al. [4] found that stobadine efficiently competed with DMPO in trapping OH• radicals generated in a Fenton-type reaction. From the concentration dependence study presented by these authors an estimate of the second-order rate constant for the reaction of stobadine with OH• was made, giving the value of $1.7 \times 10^{10} \cdot M^{-1} \cdot s^{-1}$, which is in good agreement with our results (Table II).

The potency of stobadine to prevent ethylene production from KMBA, characterized by IC_{50} value, was compared for 3 different OH•-generating systems: a chemical system, an enzymatic system, and a membrane-bound enzymatic system. As shown in Table I closely related IC_{50} values were obtained for all three systems studied, suggesting that stobadine is an efficient scavenger of OH• radicals produced not only in a free solution but also in a membrane. However, this hypothesis needs further corroboration since KMBA is not a strictly specific scavenger of OH• radicals [11].

In conclusion, these studies demonstrate that stobadine is a potent OH• radical scavenger. The ability of stobadine to scavenge free radicals may contribute to its cardioprotective properties.

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