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Superoxide Radical Scavenging by Phenolic Bronchodilators under Aprotic and Aqueous Conditions

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ABSTRACT. Asthmatic airway disease is accompanied by the appearance of inflammatory cells which produce reactive oxygen species (ROS). Therefore, the radical scavenging properties of the bronchodilators reproterol, fenoterol, salbutamol and terbutaline toward superoxide anion radicals and hydroperoxyl radicals were investigated in a model system by electron paramagnetic resonance spectroscopy (EPR) and photometric approaches. The substances under study showed activity in superoxide radical scavenging under aprotic and protic conditions as well. The efficiency of the reaction decreased in the order: fenoterol > salbutamol > reproterol > terbutaline > oxyfedrine when DMSO was used as an aprotic solvent. In an aqueous system, the rate constants decreased in the order: fenoterol > reproterol > salbutamol. It is suggested that the antioxidant effect of these β_2-agonists is an additional advantage in treatment of asthmatic lung disease, reducing the negative consequences of airway inflammation. BIOCHEM PHARMACOL **56**;3:301–305, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. asthma; beta-agonists; bronchodilators; meta-diphenols; superoxide radicals; EPR

Bronchial hyperresponsiveness indicated by eosinophilia and resulting in chronic inflammatory processes is characteristic of asthmatic airway obstruction. Whereas contraction of bronchial smooth muscle was previously considered the most important contributing factor, it subsequently became clear that edema of the airway wall as a result of microvascular leakage and luminal exuded airway secretions due to inflammatory changes contribute as well [1].

In the asthmatic airways, inflammatory cells were found to release mediators which exaggerate the asthmatic state. Among these, ROS¶ were found to play a prominent role in inflammation [1]. Apart from cellular protein oxidation and lipid peroxidation processes, increased vascular permeability is assumed to be evoked by oxygen radicals [2]. The latter pathophysiology is now judged to be as important in principle as contraction of bronchial smooth muscles in chronic airway obstruction.

In addition to their bronchodilatory action in asthma, β-agonistically active substances of diphenolic structure may offer radical scavenging properties toward ROS. In order to prove whether direct reaction with oxygen radicals is possible, we investigated here potential radical scavenging properties of bronchodilatory active substances of phenolic structure against superoxide anion radicals ($O_2^{\cdot -}$) and perhydroxyl radicals ($HO_2^{\cdot}$) in a model system. Reproterol, fenoterol, and terbutaline were used as meta-diphenol (resorcinol) derivatives, whereas salbutamol and oxyfedrine were bronchodilators of related structure (Scheme 1). Because these substances can be dissolved in lipophilic as well as in hydrophilic environments, we studied superoxide scavenging properties in aqueous solution and in an aprotic medium as well, using DMSO to simulate an aprotic environment. EPR, as well as UV-absorption spectroscopy, were applied to detect directly the scavenging of superoxide radicals in an aprotic medium. Spin-trap experiments were performed to study radical reactions in aqueous solution.

MATERIALS AND METHODS

Salbutamol, fenoterol, and terbutaline were from Sigma or supplied by Asta Medica. Reproterol and oxyfedrine were gifts from Asta Medica. Potassium superoxide and crown ether 18-C-6 were purchased from Fluka, and DMSO (anhydrous, under nitrogen) from Aldrich. Adriamycin, desferrioxamine, NADH, xanthine, and xanthine oxidase were obtained from Sigma, superoxide dismutase from Serva, and cytochrome P-450 reductase from Oxford Bio-

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¶ Abbreviations: DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; EPR, electron paramagnetic resonance; and ROS, reactive oxygen species.

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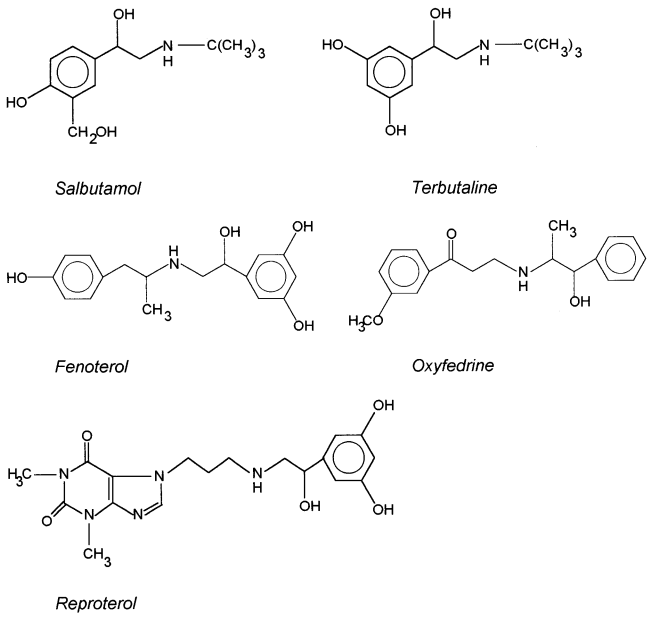

Scheme 1. Structures of investigated compounds.

medical Research, Inc. DMPO was purchased from OMRF Spin Trap Source.

Superoxide Solutions in DMSO

Superoxide anion radicals were generated in dry DMSO from potassium superoxide in the presence of equimolar amounts of crown ether 18-C-6. Concentrations of potassium superoxide and crown ether were 1 mM. All solutions were bubbled with dry N_2 before use. Subsequent work was carried out excluding access of air. For generation of the physiological radical $HO_2^{\cdot}$ by protonation of $O_2^{\cdot -}$, tridistilled water was added for a final concentration of 10 mM H_2O.

EPR Measurement of the Reaction of Superoxide with Phenolic Compounds in DMSO

One-mM solutions of superoxide in dry DMSO or DMSO/10 mM H_2O were rapidly mixed with 1-mM solutions of the phenolic compound (in dry DMSO) and then quickly frozen with liquid nitrogen using cylindrical quartz cells with 3 mm i.d. The time between mixing and cooling was 7 sec to allow reaction at room temperature. EPR measurements were then carried out at 78 K with a Bruker ESP 300E instrument at 9.5 GHz, field modulation 100 kHz, sweep width 500 G, and modulation amplitude 1 G. The reaction with superoxide was evaluated from the decay of the EPR signal characteristic of the superoxide anion radical.

In attempts to detect transient radicals expected from the reaction of phenolic compounds with superoxide or hydroxyl radicals, a continuos flow cell (Wilmad) was used at room temperature in connection with the EPR spectrometer. The flow of reactants was controlled by motor-driven double syringes of a Harvard Instruments type 22 device (Harvard Instruments) with maximum flow rates 100 mL/min.

UV Detection of $O_2^{\cdot -}$ in DMSO

The decrease in $O_2^{\cdot -}$ absorption at 270 nm was recorded in a stopped flow photometer (Applied Photophysics) at room temperature after mixing of superoxide solutions with solutions of the phenolic compounds (both 1 mM in DMSO). Time constants were calculated by bi-exponential regression functions.

Reaction with Superoxide in Aqueous Media as Studied by Spin Trapping

Superoxide radicals in aqueous solution were generated by cytochrome P-450 reductase and detected by EPR using DMPO as spin trap. Typical sample volumes of 50 μL contained: 0.0005 U of NADPH-cytochrome P-450 reductase, 0.2 mM NADPH, 50 μM adriamycin, 0.2 M DMPO, and 4% (v/v) DMSO in 0.3 M sodium phosphate buffer, pH 7.4. Concentrations of the phenolic compounds were 1–5 mM. Impurities by traces of transition metals were removed by passing the buffer through a Chelex 100 column and by addition of 0.1 mM desferrioxamine.

The spin adduct of DMPO with superoxide radicals (DMPO-OOH) was detected in a Bruker ESP 300E EPR spectrometer at room temperature. Spectra were recorded with the following instrument settings: center field: 3477 G, sweep width: 80 G, modulation frequency: 100 kHz, modulation amplitude: 0.6 G, receiver gain: $5 \cdot 10^5$, conversion time: 327.68 msec, time constant: 327.68 msec, microwave frequency: 9.77 GHz. The interpretation of spectra and the quantitative determination of rate constants for the reaction of superoxide radicals with phenolic compounds were performed as described by Dikalov *et al.* [3] for the reaction of thiols with $O_2^{\cdot -}$.

RESULTS

Reaction with Superoxide Radicals in Aprotic Media

In solutions of superoxide in DMSO, an EPR signal with $g_{II} = 2.0892$ and $g_{\perp} = 2.0072$ was observed at 78 K which is characteristic of the superoxide radical anion (Fig. 1). After addition of phenolic compounds to the superoxide solution at room temperature followed by rapid freezing, the superoxide signal was reduced dependent on scavenger concentration, unambiguously demonstrating the reaction of the superoxide radical with the phenolic compounds under study. For all investigated substances, nearly two equivalents were needed for complete quenching of the superoxide signal. This ratio was found in dry DMSO as well as in the presence of 10 mM water (Table 1).

In these experiments, no radical reaction products of the phenolic compounds were detected after freezing. In addi-

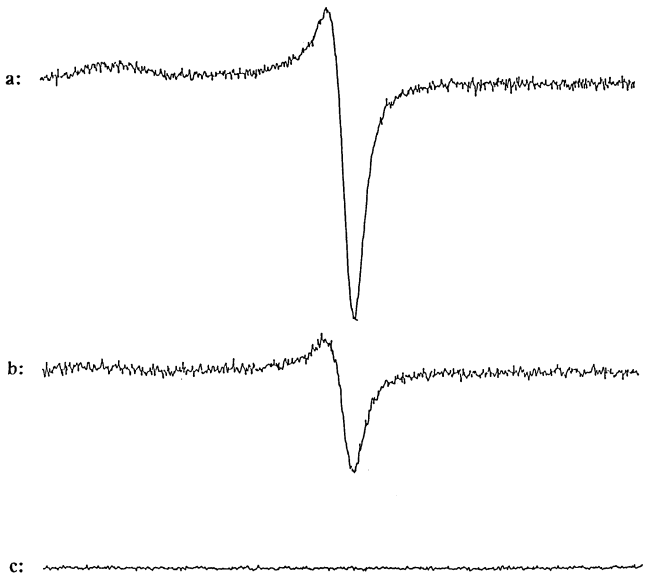

FIG. 1. (a) 1 mM superoxide in dry DMSO at 78 K showing a characteristic EPR spectrum with the anisotropic g-tensors $g_{II} = 2.0892$ and $g_{\perp} = 2.0072$. (b) Decrease in the amplitude after addition of 1 equivalent fenoterol. (c) Complete suppression of superoxide anion signal after addition of 2 equivalents fenoterol.

tional EPR continuos flow experiments, there was no evidence of the formation of transient free radicals generated from the phenolic compounds under these conditions, very probably due to their short lifetime.

The scavenging of superoxide by the investigated compounds was confirmed by UV stopped flow kinetic experiments performed to reveal the decrease in the absorption of $O_2^{\cdot -}$ at 270 nm after mixing with the phenolic compound (Fig. 2). The second order rate constants of the reactions are shown in Table 2. With salbutamol, in addition to the intensity decrease for $O_2^{\cdot -}$, the formation of a product at 320 nm was observed with a rate constant $k_3 = 120\ M^{-1}\ sec^{-1}$. The signal decay was fitted in a bi-exponential way, resulting in two reaction constants. The activity decreased in the order: fenoterol > salbutamol > reproterol ≫ terbutaline [DMT] oxyfedrine for k_1 and fenoterol ≫ reproterol > terbutaline > salbutamol > oxyfedrine for k_2, respectively, and was independent of the water content of the sample. Initial rate constants (k_1) were approximately tenfold higher than the k_2 values (except for salbutamol, whose k_1 value was 150-fold higher).

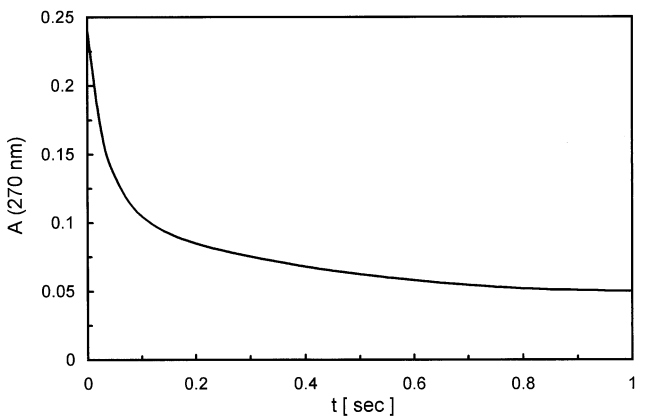

FIG. 2. Decay of the absorbance at 270 nm after addition of fenoterol to superoxide, both 1 mM in dry DMSO at room temperature (UV-VIS-stopped-flow spectra).

Reactions with Superoxide in Aqueous Media

The compounds fenoterol, salbutamol and reproterol, which were the most effective scavengers of $O_2^{\cdot -}$ in DMSO and DMSO/water, were also investigated in aqueous buffer. In this system, the scavenging properties for $O_2^{\cdot -}$ were studied indirectly using the spin trap DMPO. Direct detection of $O_2^{\cdot -}$ by freezing techniques as used for DMSO was not applicable in this case due to rapid dismutation of $O_2^{\cdot -}$ in an excess of water.

The calculated rate constants (k_{SC}) for fenoterol, reproterol, and salbutamol resulted in 1470 $M^{-1}\ sec^{-1}$, 840 $M^{-1}\ sec^{-1}$ and 100 $M^{-1}\ sec^{-1}$. These constants were means of N = 5 determinations with no more than 20% SD. The rate constants obtained in this system were in the same range (10^2–$10^3\ M^{-1}\ sec^{-1}$) as the values calculated for DMSO or DMSO/10 mM water (Table 2, k_1 values).

DISCUSSION

The results reveal that fenoterol, reproterol, salbutamol, terbutaline, and oxyfedrine react with superoxide in an aprotic medium. The presence of small amounts of water was without remarkable effect. Because protonation of superoxide in the presence of water should lead to the generation of the more reactive perhydroxyl radical, this behavior differs from the reaction of other compounds, e.g. lipids. Lipid peroxidation is brought about only by perhydroxyl radicals, but not with the unprotonated superoxide anion radical [4–6].

In aqueous buffer solution, the phenolic substances show rather similar rate constants compared to an aprotic medium. This finding may indicate the same mechanism for the reaction of the phenolic substances with superoxide anion and hydroperoxyl radicals. In a first step, it is likely that unstable phenoxyl radicals or, in the case of the

TABLE 2. Rate constants for the reaction of 1 mM superoxide with 1 mM phenol compound in A: dry DMSO and B: DMSO containing 10 mM water. The constants k_1 and k_2 refer to the biphasic signal decay of the superoxide absorption at 270 nm.

	$k_1[M^{-1}\ sec^{-1}]$		$k_2[M^{-1}\ sec^{-1}]$	
	A	B	A	B
Fenoterol	437.6	439.9	48.3	47.0
Salbutamol	232.3	235.1	1.4	1.5
Reproterol	187.2	188.2	8.5	8.5
Terbutaline	32.7	31.8	2.9	2.9
Oxyfedrine	21.7	21.9	0.9	0.9

TABLE 1. Equivalents of bronchospasmolytic agents needed for complete quenching of the EPR signal derived from 1 mM superoxide in dry DMSO and DMSO containing 10 mM water, respectively.

	Terbutaline	Salbutamol	Oxyfedrine	Fenoterol	Reproterol
DMSO	1.9	1.5	2.1	1.9	1.9
DMSO/10 mM H_2O	1.9	1.6	2.0	2.0	1.8

resorcinol derivatives, meta-semiquinone radicals are formed which, however, were not detectable under our conditions. These intermediates may react further, undergoing cyclization of the aliphatic side-chain as described for catecholamines [7, 8]. The formation of intermediate products and the appearance of side reactions may also be responsible for the biphasic signal decay observed in UV-spectroscopy. The apparent k_2 values are small compared to k_1 and negligible with the exception of fenoterol. The slower decay of the absorption curve might be explained by secondary reactions leading to an overlapping of absorption spectra.

Because the majority of patients use the bronchodilators in the form of aerosol, interaction within the lining fluid of the bronchial epithelium and its surface is important. Whereas the bronchial epithelial surfaces are the first targets, penetration into more apolar compartments is facilitated by the solubility behavior of these phenolic compounds. Partitioning experiments in octanol/water revealed a value of 0.39 for the partitioning coefficient for reproterol [9] and a value of 0.83 for fenoterol,* meaning that a remarkable amount is distributed in the apolar phase. In anhydrous DMSO, the superoxide $O_2^{\cdot -}$ undergoes no protonation, and decay due to dismutation via its protonated form $HO_2^{\cdot}$ is thereby suppressed. Consequently, $O_2^{\cdot -}$ is a long-lived species under these conditions. A similar situation may be found in aprotic regions of biologic membranes. Accordingly, a steady-state content of $O_2^{\cdot -}$ in intact plant tissues has been reported [10], and aprotic sites for the storage of $O_2^{\cdot -}$ *in vivo* have been considered [10]. Thus, the scavenging of superoxide in aprotic regions of the membrane might play a role in the physiological action of these phenolic compounds.

Interaction of phenolic compounds with cell membranes resulting in changes in membrane fluidity [11, 12] might be an additional effect.

The oxygen radical scavenging properties of these β-agonistic substances seem to be slight in comparison with cellular antioxidant defense systems such as glutathione ($k_{SC} = 1.8 \cdot 10^5\ M^{-1}\ sec^{-1}$) or vitamin E ($k_{SC} = 10^5\ M^{-1}\ sec^{-1}$) [3]. However, local concentrations of the drug could be high enough to provide an additional protection of the cell from oxygen radical damage. Recently, Gillissen *et al.* [13, 14] were able to demonstrate such an antioxidant effect of β_2-agonists in cell cultures. The dosage incorporated by the bronchial system through inhalation frequently surpasses the range of subnanomolar concentrations necessary for agonism at specific binding sites. For this reason, other nonspecific sites may be important for anti-asthmatic as well as anti-inflammatory efficiencies. Therefore, we suggest that the antioxidant/antiinflammatory effect of the studied β_2-agonists may represent an additional advantage in the treatment of asthmatic lung disease.

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