Antioxidative Action of the β-Adrenoceptor Antagonist Bopindolol and Its Metabolite 18-502

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ABSTRACT—The antioxidative effects of β-adrenoceptor antagonists and related compounds were investigated. Among the β-adrenoceptor antagonists, the agents with a potent membrane-stabilizing activity such as bopindolol and propranolol strongly inhibited the hydrogen peroxide (H2O2)-induced lipid peroxidation of liver microsomes. Fifty percent inhibition concentration values for the lipid peroxidation of bopindolol, 18-502 (metabolite of bopindolol) and propranolol were calculated to be 1.8 μM, 10 pM and 2.3 μM, respectively. The same potency order of the agents for the inhibition of lipid peroxidation was observed in rat heart homogenates. Furthermore, cytochrome P-450-catalyzing lipid peroxidation in microsomes and H2O2-induced lipid peroxidation in coronary arteries or cardiac muscles of pigs were also inhibited by bopindolol, whereas propranolol was less effective. Bopindolol and 18-502, but not propranolol, scavenged a stable free radical 1,1-diphenyl-2-picrylhydrazyl. Thus it was concluded that bopindolol that has membrane-stabilizing and radical scavenging activities is a more potent antioxidant than propranolol and may produce a beneficial effect for the treatment of ischemic cardiac diseases.

Keywords: β-Adrenoceptor antagonist, Antioxidant, Bopindolol, Lipid peroxidation, Radical scavenger

Bopindolol [4-(2-benzoyloxy-3-tert-butylaminopropoxy)-2-methyl indole hydrogen malonate] is a new nonselective β-adrenoceptor antagonist with potent membrane-stabilizing activity that is used as an antihypertensive agent or in the treatment of angina pectoris (1–6). Evidence indicates that some β-adrenoceptor antagonists show an antioxidative effect on lipid peroxidation, and a membrane-stabilizing activity may contribute to the prevention of lipid peroxidation (7–10). In considering that ischemia-reflow of tissue organs can generate reactive oxygen species which lead to tissue damage (11–13), agents with an antioxidative effect are expected to be favorable for the treatment of ischemic diseases. Indeed, our previous report showed that hydrolyzed bopindolol (18-502), which is a metabolite of bopindolol with β-adrenoceptor antagonist action, has a protective effect via antioxidant action against the contractile dysfunction following reperfusion after myocardial ischemia (14). In the present study, we investigated in detail the antioxidative or radical scavenger action of bopindolol and 18-502 as well as that of propranolol.

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MATERIALS AND METHODS

Chemicals
Reagents used in the experiments were obtained from the following sources: Bopindolol [4-(2-benzoyloxy-3-tert-butylaminopropoxy)-2-methyl indole hydrogen malonate] and 18-502 [4-(3-tert-butylamino-2-hydroxypropoxy)-2-methyl indole] from Sandoz Pharmaceuticals (Tokyo), dl-propranolol from Nacalai Tesque (Kyoto) and hydrogen peroxide from Santoku Kagaku (Tokyo). Hypoxanthine, xanthine oxidase, diethylenetriamine pentaacetic acid (DETPAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) were from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Measurement of lipid peroxidation in liver microsomes and tissue homogenates
Liver microsomes (0.5 mg/ml) prepared from nontreated male Sprague-Dawley rats (150–300 g), as described previously (15), were incubated with β-adrenoceptor antagonists or related compounds in the presence or absence
of hydrogen peroxide (H₂O₂) in 0.05 M Tris-HCl buffer (pH 7.4) at a total volume of 1 ml at room temperature for the indicated times and then centrifuged at 105,000 × g for 60 min to remove residual agents. Lipid peroxides in the pellets, which were resuspended in the same buffer, were measured by the method of Buege and Aust (16), with some modifications (17), as thiobarbituric acid reactive substances (TBARS) and calculated as malondialdehyde (MDA) equivalents from the molecular coefficient of 1.56 × 10⁵ M⁻¹ cm⁻¹. In measuring enzyme dependent lipid peroxidation, the microsomes were incubated with agents in the presence of a NADPH generating system (15) for 30 min at room temperature, and then TBARS was measured.

When necessary, the rat was decapitated and the heart was removed after perfusion with 1.15% potassium chloride via the inferior vena cava. The isolated heart was homogenized with a glass homogenizer in 5 volume of ice-cold 0.05 M Tris-HCl buffer (pH 7.4), and the TBARS in the heart homogenates was measured after incubation with 3.3 mM hydrogen peroxide in the presence or absence of the agents for 30 min at room temperature.

Moreover, the coronary arteries and cardiac muscles isolated from pig hearts, which were obtained from a slaughterhouse, were cut into segments and incubated with or without agents in Krebs-Henseleit solution at 37°C for 40 min. After washing with the solution, the tissues were further incubated with 3.3 mM H₂O₂ in 0.05 M Tris-HCl buffer (pH 7.4) for 30 min at room temperature. The residual H₂O₂ in the reaction mixtures was discarded and then tissues were homogenized with a polytron homogenizer (Kinematica, Lucerne, Switzerland) in 2 or 5 volumes of the ice-cold buffer. Thus the TBARS in the homogenates was measured. Protein concentrations in tissue homogenates and microsomes were measured by the method of Lowry et al. (18).

Radical-trapping experiments

The radical trapping ability of the agents for the stable radical DPPH was estimated as follows: The test agent was dissolved in water. A blank assay was performed in the same manner using only water instead of the agent solution. DPPH (100 μM) dissolved in ethanol was mixed with an equal volume of test agents (0.01—10 mM) for 40 sec, and the electron spin resonance (ESR) spectra were recorded at room temperature in a quartz flat cell with a JES-REIX spectrometer operating at X band (9.415 GHz) and a modulation frequency of 100 kHz, microwave power of 8 mW, receiver gain of 1.6 × 10⁴, field modulation width of 0.79 × 0.1, sweep time of 2 min, and time constant of 0.1 sec. The central field ± sweep width was set at 335 ± 10 mT. The relative DPPH radical intensity height was calculated to divide the signal height of DPPH by that of simultaneously recorded Mn²⁺. The scavenging ability of an agent for a DPPH radical was estimated by comparing the relative intensity height between a test drug assay and the blank assay.

Radical-trapping abilities of β-blockers for superoxide anion (O₂⁻) and hydroxyl radical (·OH) were determined with DMPO as a radical-trapping agent as described previously (19). The test agent dissolved in water was exposed to the free radical generation system, hypoxanthine-xanthine oxidase for O₂⁻ and H₂O₂/FeSO₄ for ·OH; and the formation of the radical spin adduct DMPO-OOH and DMPO-OH was monitored under the previously described ESR-spectrometer settings.

Statistical analyses

Data are presented as means ± S.D. Statistical analyses were performed by Student’s t-test, and P values < 0.05 were taken as significant.

RESULTS

Inhibition of H₂O₂-induced lipid peroxidation of liver microsomes by β-adrenoceptor antagonists and related compounds

To evaluate the antioxidant action of β-adrenoceptor antagonists and membrane stabilizing agents, various agents were examined using H₂O₂-induced lipid peroxidation of liver microsomes. As shown in Table 1, propranolol and bopindolol markedly inhibited the lipid peroxidation (79% and 82% inhibition, respectively), whereas labetalol, metoprolol and atenolol moderately inhibited it. Acebutol was not inhibitory. In the cases of membrane stabilizing agents, the lipid peroxidation was decreased to 25% by quinidine and to 34% by procaine. Moderate inhibition of the peroxidation was observed by lidocaine.

Table 1. Effects of β-adrenoceptor antagonists on H₂O₂-induced lipid peroxidation of rat liver microsomes

| Addition     | H₂O₂ (0.75 mM) | TBARS (nmol/mg) | % of Control |
|--------------|----------------|-----------------|-------------|
| Control      | −              | 0.263 ± 0.27    | 100         |
| +            | 1.322 ± 0.083  |                 |             |
| Propranolol  | +              | 0.277 ± 0.053   | 21          |
| Acebutolol   | +              | 1.537 ± 0.240   | 116         |
| Bopindolol   | +              | 0.242 ± 0.061   | 18          |
| Labetalol    | +              | 0.881 ± 0.039   | 66          |
| Metoprolol   | +              | 1.123 ± 0.067   | 85          |
| Atenolol     | +              | 1.138 ± 0.069   | 86          |

The liver microsomes were incubated with H₂O₂ (0.75 mM) together with or without a β-adrenoceptor antagonist (1 mM) at room temperature for 30 min. Values represent means ± S.D. for three incubations.
Since bopindolol and propranolol showed potent antioxidant actions on the lipid peroxidation of liver microsomes, the agents including hydrolyzed bopindolol (18-502) were used in further studies. The values of the fifty percent inhibition concentration (IC50) of bopindolol, propranolol and 18-502 for the lipid peroxidation of liver microsomes were calculated to be 1.8 \(\mu\)M, 2.3 \(\mu\)M and 10 \(\mu\)M, respectively (Fig. 1). When each agent was incubated with microsomes at the IC50, the inhibitory effect on the lipid peroxidation was observed after 20 min and reached its plateau at 30 min (Fig. 2).

In the case of lipid peroxidation induced via the liver microsomal cytochrome P-450 system in the presence of the NADPH-generating system, the IC50 values of bopindolol and propranolol were 4 \(\mu\)M and 100 \(\mu\)M, respectively. Thus it was clarified that bopindolol inhibits lipid peroxidation induced not only by \(H_2O_2\) but also by the enzyme.

Inhibition of \(H_2O_2\)-induced lipid peroxidation of heart and arteries homogenates

As shown in Fig. 3, bopindolol, 18-502 and propranolol inhibited lipid peroxidation of rat heart homogenates, and the IC50 values were 42 \(\mu\)M, 108 \(\mu\)M and 251 \(\mu\)M, respectively. Bopindolol (0.1 mM and 1 mM) also inhibited the lipid peroxidation in pig coronary arteries (41%...
and 55% inhibition) and cardiac muscles (22% and 33% inhibition) (Table 3). Under the same conditions, pranolol did not show any effect on the peroxidation of the pig heart tissues.

**Radical trapping effect**

When bopindolol was allowed to react with the stable free radical DPPH, the radical was scavenged in a dose-dependent manner (0.01–2 mM), showing a 60% trapping of the radical at 1 mM. The same concentration (1 mM) of 18-502 showed a 40% trapping of DPPH radicals, but propranolol had no effect (Figs. 4 and 5).

Bopindolol, 18-502 and propranolol did not show any effect on the formation of radical spin adducts of \( \text{O}_2^- \) and of \( \cdot \text{OH} \) (Fig. 6).

**DISCUSSION**

To evaluate the antioxidative action of \( \beta \)-adrenoceptor antagonists, various drugs were examined. Among the \( \beta \)-adrenoceptor antagonists used in these experiments, propranolol and bopindolol markedly inhibited \( \text{H}_2\text{O}_2 \)-induced lipid peroxidation of liver microsomes. Considering that the potential of membrane-stabilizing activity of the agents is in the order of propranolol > acebutolol > labetalol, metoprolol > atenolol (20) and that bopindolol also has a potent membrane-stabilizing activity (1, 2), it is clear that the inhibitory action against lipid peroxidation by a \( \beta \)-adrenoceptor antagonist depends on its membrane-stabilizing activity. Moreover, the typical membrane-stabilizing agents quinidine and procaine also inhibited the \( \text{H}_2\text{O}_2 \)-induced lipid peroxidation, with potencies comparable to that of propranolol, supporting that the membrane-stabilizing activity of these agents contributes to inhibition of membrane lipid peroxidation. Thus we investigated in detail the antioxidant effect of propran-

### Table 3. Effect of bopindolol on lipid peroxidation in porcine coronary artery and cardiac muscle

| Tissues            | Concentration mM | Control       | Bopindolol  |
|-------------------|-----------------|---------------|-------------|
| Coronary artery   | 0.1             | 0.128±0.043   | 0.076±0.008** (59) |
|                   | 1               | 0.092±0.053   | 0.041±0.027** (45) |
| Cardiac muscle    | 0.1             | 0.047±0.029   | 0.037±0.023** (78) |
|                   | 1               | 0.055±0.028   | 0.037±0.02* (67) |

Arteries and muscles were incubated with bopindolol (0.1 mM, 1 mM) at 37°C for 40 min followed by treatment with \( \text{H}_2\text{O}_2 \) (3.3 mM) at room temperature for 30 min. TBARS in the homogenates were measured. Values represent means±S.D. for 5 to 6 experiments. The value in the parenthesis shows the percent of the control. *P<0.05, **P<0.01: control vs treated.

![Fig. 4](image-url)  
**Fig. 4.** Effects on \( \beta \)-adrenoceptor antagonists on the ESR signals formed by DPPH. DPPH (100 \( \mu \)M) in ethanol was mixed with the agents at the indicated concentrations, and the ESR signals for DPPH radicals after 40 sec were monitored as described in Materials and Methods (g=2.006, a\( ^\beta \)=0.876 mT).

![Fig. 5](image-url)  
**Fig. 5.** Determination of DPPH radical scavenging activity of agents by ESR. The DPPH radical scavenging effect of agents was calculated from the ESR signals shown in Fig. 4 and the height of the asterisked signal of the control was used as 100% of DPPH. ○, bopindolol; △, 18-502; ●, propranolol.
olol, bopindolol and its metabolite 18-502. Judging from the IC\textsubscript{50} values of the three agents for H\textsubscript{2}O\textsubscript{2}-induced lipid peroxidation in liver microsomes, bopindolol (IC\textsubscript{50}, 1.8 \textmu M) was the most potent inhibitor of the peroxidation. Furthermore, bopindolol also inhibited H\textsubscript{2}O\textsubscript{2}-induced lipid peroxidation in rat heart homogenates (IC\textsubscript{50}, 42 \textmu M), and the inhibitory effect of bopindolol was greater than that of propranolol. This IC\textsubscript{50} value of bopindolol for the lipid peroxidation is the smallest among those for all the \beta-blocking agents as seen in the literature, and only carvedilol with an IC\textsubscript{50} value of 8.1 \textmu M for lipid peroxidation of rat brain homogenates is comparable with bopindolol (7, 9, 10).

Since the cytochrome P-450 system in microsomes can generate superoxide anion and hydrogen peroxide by receiving electrons via NADPH (21, 22), this was examined as an enzyme catalyzed lipid peroxidation. The fact that bopindolol inhibits the enzyme-derived lipid peroxidation suggests that bopindolol may inhibit endogenously generated lipid peroxidation as seen in oxi-

Fig. 6. Effects of \beta-adrenoceptor antagonists on the ESR signals formed via hypoxanthine/xanthine oxidase (A) and H\textsubscript{2}O\textsubscript{2}/FeSO\textsubscript{4} (B). A: hypoxanthine (500 \textmu M), xanthine oxidase (0.02 unit) and DMPO (230 \textmu M) were mixed in 0.1 M phosphate buffer (pH 7.8) in the presence of DETAPAC (960 \textmu M) with the indicated agents at room temperature, and the ESR signal for DMPO-OOH after 40 sec was monitored as described in Materials and Methods (g=2.006, a\textsubscript{N}=1.418 mT, a\textsubscript{H}=1.142 mT, a\textsubscript{S}=0.137 mT). B: FeSO\textsubscript{4} (250 \textmu M), H\textsubscript{2}O\textsubscript{2} (250 \textmu M) and DMPO (92 \textmu M) were mixed in 0.1 M phosphate buffer (pH 7.8) in the presence of DETAPAC (250 \textmu M) with the indicated agents, and the ESR signal for DMPO-OH after 40 sec was monitored (g=2.007, a\textsubscript{N}=1.488 mT, a\textsubscript{H}=1.488 mT).
dative stress such as ischemia/reperfusion. Indeed, we reported that nicorandil with inhibitory action against \( \text{H}_2\text{O}_2 \)-induced lipid peroxidation has improved oxidative changes seen after ischemia/reperfusion of isolated rat liver (23).

When the isolated pig coronary arteries and cardiac muscles were incubated with bopindolol or propranolol followed by \( \text{H}_2\text{O}_2 \)-treatments, the lipid peroxidation in each homogenate was inhibited only by bopindolol but not by propranolol. Thus bopindolol may have an affinity to heart and coronary arteries. Probably the lipophilicity of bopindolol may be involved in its affinity because the bezoyloxy group of the bopindolol molecule enhances its lipophilicity compared with propranolol (Fig. 7), increasing its interaction with membrane lipids. It is, therefore, presumed that bopindolol is accumulated in biological membranes and then interferes with the \( \text{H}_2\text{O}_2 \)-derived lipid peroxidation of membranes. However, it can not be excluded that other factors may contribute to the preferential protection of bopindolol against lipid peroxidations in heart and coronary arteries.

When radical scavenging action was examined using an electron spin resonance (ESR) spectrometer, bopindolol, and less effectively 18-502, reduced the stable free radical DPPH, but neither hydroxyl radical nor superoxide anion was scavenged by the agents. Propranolol could not scavenge any radicals. Since DPPH is known to abstract labile hydrogen atoms (24, 25), it was assumed that the hydrogen atom in the indole moiety of bopindolol reacts with the DPPH radical, whereas propranolol with the naphthalene moiety fails to scavenge the DPPH radical. The indole moiety of 18-502 can also trap DPPH radicals. As reported elsewhere (19, 26, 27), in which the scavenging ability for DPPH radicals may be connected to an inhibitory action on lipid peroxidation, the inhibitory action of bopindolol against lipid peroxidation may be strengthened by its ability to trap radicals formed during membrane peroxidation. Although we measured the DPPH radical scavenging activity of all drugs used for experiments on lipid peroxidation, none of the drugs except for bopindolol and 18-502 could trap the radical. Taking these results into account, it is considered that propranolol exhibits its antioxidant action via a membrane stabilizing activity, whereas the antioxidant activity of bopindolol, and also that of 18-502, is due to both a membrane stabilizing activity and free radical scavenging activity. Thus bopindolol is a more potent antioxidant than propranolol.

Reactive oxygen species have been proposed to play a role in the production of ischemic myocardial injury (12, 13), evidence suggesting that lipid peroxidation of biological membranes caused by oxygen free radicals alters membrane fluidity and permeability, resulting in a loss of cell function (28, 29). Thus the agents with activity for reducing lipid peroxidation and radical formation can improve ischemic myocardial injury. Our previous report indicated that 18-502 has a cardioprotective action against contractile dysfunction produced by coronary stenosis and reperfusion in dogs, and the antioxidant action of 18-502 was suggested to contribute to its protective action (14). It is therefore strongly suggested that bopindolol and its metabolite 18-502 act as antioxidants and are beneficial for the treatment of ischemic diseases.

In conclusion, bopindolol and 18-502 show potent antioxidant action via a radical scavenging and membrane stabilizing actions, suggesting that both drugs are more beneficial for treating myocardial ischemia than propranolol. This potent antioxidant activity of bopindolol may be favorable for the treatment of other diseases caused by oxidative stress.

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