QUANTITATIVE ESTIMATION OF MYOCARDIAL FIBROSIS BASED ON RECEPTOR OCCUPANCY FOR β_2 -ADRENERGIC RECEPTOR AGONISTS IN RATS

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ABSTRACT — To develop β_2 -adrenergic receptor (AR) agonists with higher selectivity, it is essential to evaluate the cardiac side effects which are the most serious side effects of this class of drugs. We studied receptor occupancy of β_1 -ARs in rats as a possible cause for the side effect of β_2 -AR agonists, namely myocardial fibrosis. Myocardial fibrosis in rats was observed on Day 7 after the administration of salbutamol and terbutaline, both of which are selective β_2 -AR agonists, at higher dose levels. To evaluate receptor occupancy, plasma concentrations of (*R*)-salbutamol and (*R*)-terbutaline, plasma protein binding and the EC₅₀ for chronotropic effects in rats were determined. Based on the plasma concentrations, the plasma protein binding and EC₅₀, receptor occupancy-time profiles were constructed. The relationship between the receptor occupancy-time profile under the curve, the AUC Φ , and the degree of myocardial fibrosis was evaluated with a multiple correlation analysis. Myocardial fibrosis was significantly correlated ($r^2 > 0.78$) to the AUC Φ with the threshold above approximately 50%, but not to plasma concentrations. These results indicate that the receptor occupancy theory is also useful for the evaluation of the chronotropic side effects of β_2 -AR agonists.

KEY WORDS: β_2 -adrenergic receptor agonists, Receptor occupancy, Myocardial fibrosis

INTRODUCTION

β_2 -adrenergic receptor (AR)s are subdivided into β_1 -, β_2 - and β_3 -subtypes and are widely distributed in cardiac muscle, adipose tissue, bronchi smooth muscle, gastrointestinal tract, blood vessels and uterus. β_2 -AR is involved in the relaxation of smooth muscle in the bronchi, gastrointestinal tract, blood vessels and uterus, and thus selective β_2 -AR agonists are widely used for the treatment of asthma and premature birth. Salbutamol and terbutaline were the first selective β_2 -AR agonists in general clinical use. These compounds were the first to be shown to produce significant attenuation of the cardiostimulant effect and to confirm the subdivision of β -ARs into β_1 and β_2 (Waldeck, 2002). However, at high dose levels in rats, they have been reported

to produce toxicity including myocardial necrosis and fibrosis (Magnusson and Hansson, 1973; Libretto, 1994). Inotropic and chronotropic responses are mainly mediated by β_1 -AR which predominates in the cardiac muscle of most experimental animals rather than β_2 -ARs (Vago *et al.*, 1984; Summers *et al.*, 1987). In the initial process for the development of highly selective β_2 -AR agonists, myocardial necrosis and fibrosis should be assessed as the target toxicity.

Recently, the receptor occupancy theory has been developed to assess the adverse/toxic effects of various drugs (Sawada *et al.*, 1997). This theory has been successfully applied to various disorders associated with related receptors, such as sleep disorders, by the blockade of β_2 -ARs and/or serotonin receptors (Yamada *et al.*, 1995a).

In the present study, in order to assess the myocardial fibrosis produced by β_2 -AR agonists by using receptor occupancy, we evaluated the relationship between the peripheral β_1 -AR receptor occupancies of (*R*)-salbutamol or (*R*)-terbutaline and the area of myocardial fibrosis in the heart after drug treatment in rats. We demonstrated the existence of a threshold above which myocardial fibrosis occurs.

MATERIALS AND METHODS

Drugs and solutions

Salbutamol sulfate (salbutamol), terbutaline sulfate (terbutaline) and *dl*-isoproterenol were purchased from Wako Pure Chemical Industries, Ltd. (minimum 98.0%, racemates with 1:1, Osaka, Japan) and dissolved in saline. Ritodrine hydrochloride, used as an internal standard, was synthesized at Kissei Pharmaceutical Co. Ltd. (Nagano, Japan). Krebs-Henseleit solution had the following composition: 118.1 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgSO_4 , 25.0 mM NaHCO_3 , 1.2 mM KH_2PO_4 and 11.1 mM glucose (pH 7.4). Other reagents and solvents used were special or HPLC grade.

Animals and treatment

All animal experiments were carried out in accordance with the Guide to the Care and Use of Experimental Animals of the Toxicological Research Lab., Kissei Pharmaceutical Co. Ltd., and were approved by the Animal Care and Use Committee of the Toxicological Research Lab., Kissei Pharmaceutical Co. Ltd. Male Sprague-Dawley rats of ages 8 weeks old and weighing 250 - 310 g were purchased from SLC (Hamamatsu, Japan). The rats were fed *ad libitum* commercial diet for rats and mice which was sterilized by γ -ray irradiation (CE-2, irradiated at 10 kGy, CLEA Japan Inc., Tokyo, Japan). Tap water, filtered and UV irradiated, was given *ad libitum* through an automatic watering system. After 7 days of quarantine and acclimation period, salbutamol (2, 6, 20 and 60 mg/kg intravenously, 30, 100, 300 and 1000 mg/kg orally) or terbutaline (2, 6, 20 and 40 mg/kg intravenously, 30, 100, 300 and 1000 mg/kg orally) were administered intravenously or orally to animals at 9:00 a.m. Control rats were dosed with saline. Blood for toxicokinetic measurement was collected from the jugular vein at 5 min, 30 min, and 1, 2, 4, 8 and 12 hr after intravenous administration, and at 30 min, 1, 2, 4, 8, 24 and 48 hr after oral administration. One week after administration, rats were exsanguinated under anesthesia with

diethyl ether and their hearts were isolated for examination for myocardial fibrosis. Eight rats were used for *in vitro* study of the chronotropic effect after 7 days of quarantine and acclimation period.

In vitro chronotropic study

Rats were killed by rapid exsanguination, and the atria were isolated and placed in Krebs-Henseleit solution. The atria were suspended in a 20-ml organ bath containing Krebs-Henseleit solution. This was maintained at 37°C and continuously gassed with a mixture of 95% oxygen and 5% carbon dioxide. A resting tension of 0.5 g was applied to the preparation at the beginning of the experiment, and the tissue was equilibrated for at least 30 min. The isometric tension generated by the tissue was measured using a force-displacement transducer (SB-1T; Nihon-Kohden, Tokyo, Japan) and recorded on a rectigraph (Rectigraph 8K; NEC San-ei, Tokyo, Japan). Initially, 10^{-6} M isoproterenol was added to the bath fluid and measured as the maximum response. After the tissue was further equilibrated for at least 30 min, concentration-response curves were constructed by cumulative addition of the appropriate drug to the bath fluid. Each preparation was exposed to only one drug. The drugs added to the bath fluid were racemates; the activity was attributed only to the (*R*)-enantiomer. The chronotropic effect on the rat heart preparation was expressed as the percentage response of the β_2 -AR against the maximum response to isoproterenol, a non-selective β -AR agonist. The EC_{50} for each drug was estimated from the concentration-response curve.

Toxicokinetics

Blood for toxicokinetics was collected as described above. The heparinized plasma was stored until analysis of (*R*)-salbutamol and (*R*)-terbutaline. (*R*)-Salbutamol concentrations were measured essentially according to the method described previously (Bergés *et al.*, 1999) using ritodrine hydrochloride as the internal standard, and (*R*)-terbutaline concentrations were also determined by the same method. Bond-Elut® Certify SPE cartridges (130 mg / 3 cc; Varian, Ca, USA) were conditioned with methanol and deionized water, and prevented from drying before applying the samples. After addition of the plasma mixture, the cartridges were washed with water as well as 1 M of acetic acid and methanol. Salbutamol and terbutaline were eluted with a mixture of chloroform and 2-propanol containing 2% ammonia. After drying the eluent, the residue was dissolved in dichloromethane contain-

ing trifluoroacetic acid. The (*R*)-enantiomer was analyzed by HPLC with a fluorescence detector at excitation and emission wavelengths of 230 and 309 nm after the (*R*)- and (*S*)-enantiomers were separated by a chiral column (chirex (*S*)-ICA and (*R*)-NEA, 250 $\times$ 4.6 mm, Phenomenex, USA). The $C_{\max}$, which was the maximum concentration after administration, was estimated from the time profile of the (*R*)-enantiomer. The area under the time-plasma concentration curve (AUC) was calculated from the time profile of the (*R*)-enantiomer by the trapezoidal rule.

Binding to Plasma Protein

To investigate protein binding, salbutamol or terbutaline was spiked into pooled rat plasma (SLC) in the range of 0.1 - 10 μ M as the (*R*)-enantiomer (active form). The plasma was incubated at 37°C for 30 min, and then 1 ml of plasma was placed in a Centrifree® (MPS-3; Amicon, Ca, USA), and centrifuged at 1500 $\times$ g for 10 min. Each drug was extracted from aliquots of the filtrate and the plasma, and the drug concentration ((*R*)-enantiomer) was measured as described in the toxicokinetic section. The adsorption of each drug on the Centrifree® was negligible. Protein binding at each concentration was measured in duplicate in two experiments, and the percentage was calculated by using the following equation:

$$\text{Protein binding (\%)} = (1 - C_f / C_t) \times 100$$

C_f : concentration of enantiomer in the filtrate

C_t : concentration of enantiomer in the plasma

Quantitative microscopic findings

The heart was removed at 7 days after drug administration, and a 3 - 5 mm part, including the papillary muscle of the right ventricle, was excised, followed by fixation with 10% phosphate buffered formalin. Three μ m thin sections were then cut and stained with Masson-trichrome. The blue-stained regions, excluding the collagen surrounding the small blood vessels and that in the intercellular space in the control rats, were measured microscopically by an image analyzer (LUZEX3, Hitachi, Tokyo, Japan) to assess the area of myocardial fibrosis.

Estimation of the area of receptor occupancy-time under the curve

Receptor occupancy of β_2 -AR agonist against β_1 -ARs was calculated by using the following equation:

$$\text{Receptor occupancy } (\Phi\%) = C \times f / (C \times f + EC_{50}) \times 100$$

$$f = 1 - \text{protein binding} / 100$$

where f was the unbound fraction of drug in plasma and C was the plasma concentration of (*R*)-enantiomer. The EC_{50} was estimated from the concentration-response curve of the chronotropic effect by β_1 -AR agonism from the β_2 -AR agonist. From the receptor occupancy-time profile, the $AUC\Phi$ (%·hr) for each animal was calculated by the trapezoidal rule for up to 12 or 48 hr after intravenous or oral administration, respectively. Furthermore, based on the designated threshold for receptor occupancy, the area under the profile above the threshold for each animal was determined by the trapezoidal rule. The $AUC\Phi$ and the area of myocardial fibrosis were plotted against the horizontal and vertical axes at the variable threshold, and each multiple correlation coefficient was calculated.

RESULTS

In vitro chronotropic effect

Salbutamol and terbutaline produced chronotropic effects on the rat atria in a concentration-dependent manner (Fig. 1). The maximum heart rate after treatment with terbutaline was as potent as that of isoproterenol, and that of salbutamol was comparable to terbutaline and isoproterenol. The EC_{50} s for (*R*)-salbu-

Fig. 1. Effects of β_2 -AR agonists on heart rate in isolated rat atria. Each point represents the mean $\pm$ standard error of 4 experiments. Half concentration of racemic drug, which was added to the organ bath, was indicated as (*R*)-enantiomer concentration. The chronotropic effect of the rat heart preparation was revealed as the response percentage of β_2 -AR agonist against a maximum response of the isoproterenol, a non-selective β -AR agonist.

tamol and (*R*)-terbutaline were 4.0 ± 3.3 and 1.8 ± 0.46 μM , respectively.

Plasma protein binding

The mean plasma protein binding of (*R*)-salbutamol and (*R*)-terbutaline was 8.7% and 14.3% in the concentration range of 0.1 to 10 μM , respectively. Differences in plasma protein binding were not observed between the (*R*)-enantiomers and (*S*)-enantiomers of both salbutamol and terbutaline. The results were in good agreement with previous reports (Morgan *et al.*, 1986; Nyberg, 1984). The mean binding value of the (*R*)-enantiomer was used to estimate the receptor occupancy.

Toxicokinetics and receptor occupancy profile

Fig. 2 (A) to (D) show the plasma (*R*)-enantiomer concentration profiles after intravenous and oral administration of salbutamol and terbutaline. In cases of intravenous administration to rats of 60 mg/kg of salbutamol and 40 mg/kg of terbutaline, plasma (*R*)-salbutamol and (*R*)-terbutaline concentration was reached C_{max} immediately after their administration, and almost all amounts of the compound were then eliminated from the plasma within 12 hr. In case of oral administration, the plasma concentrations of salbutamol and terbutaline were less than those following intravenous administration. Fig. 2 (E) to (H) show the receptor occupancy profiles derived from the plasma concentration profiles. After intravenous administration of 60 mg/kg of salbutamol and 40 mg/kg of terbutaline, the maximum occupancies reached approximately 100% immediately after dosing.

Myocardial fibrosis

Photo 1 shows typical fibrosis of the myocardial papillary muscle observed 7 days after a single intravenous administration of 60 mg/kg of salbutamol to rats. The myocardial fibrosis was localized to a region on the endocardial side of the rat heart. Fibrosis was also induced by intravenous administration of 40 mg/kg terbutaline and 20 mg/kg salbutamol, and oral administration of 300 mg/kg salbutamol and 1000 mg/kg with both drugs. However, the fibrosis was not observed by the treatment of rats with 2 and 6 mg/kg intravenously and 30 and 100 mg/kg orally of both drugs, respectively.

Correlation between myocardial fibrosis and Receptor occupancy

The C_{max} and AUC of both salbutamol and terbu-

taline were not related to the ability to produce myocardial fibrosis (data not shown). The β_1 -AR occupancy of the agonist was calculated based on the plasma concentration obtained from the toxicokinetic measurements, unbound fraction ratio and the EC_{50} obtained from the *in vitro* chronotropic study, and was plotted against the time after administration (Fig. 2, E to H). The area of myocardial fibrosis was plotted against the $\text{AUC}\Phi$ (%·hr) above the variable occupancy designed as the threshold, and multiple correlation coefficients (r^2) corresponding to the threshold were obtained. The relationship between $\text{AUC}\Phi$, β_1 -AR occupancy-time profile, without the threshold and myocardial fibrosis was not correlated ($r^2 = 0.12$, Fig. 3A). Myocardial fibrosis was significantly correlated ($r^2 = 0.78$) to $\text{AUC}\Phi$ with the threshold of 50% (Fig. 3B). Fig. 4 shows the relationship between the threshold of $\text{AUC}\Phi$ and the correlation coefficients. No correlation was observed when the threshold was up to 40% ($r^2 \leq 0.25$), whereas correlation was observed when the threshold was $\geq 50\%$.

DISCUSSION

In the present study, we examined the side effects of β_2 -AR agonists on the cardiovascular system on the basis of receptor occupancy in rats. The present results demonstrate that the myocardial fibrosis caused by two selective β_2 -AR agonists, salbutamol and terbutaline, depends on their receptor occupancy, but not on plasma drug concentrations.

Both salbutamol and terbutaline induced myocardial fibrosis in 7 days after oral or intravenous administration to rats at toxic doses, although these drugs were thought to be selective β_2 -AR agonists. The results of an *in vitro* chronotropic study indicated that both (*R*)-salbutamol and (*R*)-terbutaline stimulate β_1 -AR with EC_{50} s of 4.0 μM and 1.8 μM , respectively. The activities of salbutamol and terbutaline against β_2 -AR were approximately 400- and 200-fold greater than those against β_1 -AR, respectively, as shown in a previous study (Hochhaus and Möllmann, 1992) and the present experiment. The ratio of β_1/β_2 -AR activity for salbutamol was almost the same as that reported in a previous study (Jack, 1991), in which salbutamol possessed 500-fold greater activity toward β_2 -AR as compared to β_1 -AR. They also showed that terbutaline has an almost similar profile to salbutamol.

The activities of β -AR agonists also depend on their binding property toward β -AR. In this study, we determined plasma concentrations of the (*R*)-enanti-

Myocardial fibrosis by β -adrenergic receptor agonists.

Fig. 2. Plasma concentration- and receptor occupancy of β_1 -AR - time curves after administration of β_2 -AR agonists to rats. The left panels: the plasma concentration-time curves after the administration of salbutamol at intravenous dose of 2 ($\blacktriangle$), 6 ($\circ$), 20 ($\bullet$) and 60 mg/kg ($\triangle$) (A); salbutamol at oral dose of 30 ($\blacktriangle$), 100 ($\circ$), 300 ($\bullet$) and 1000 mg/kg ($\triangle$) (B); terbutaline at intravenous dose of 2 ($\blacktriangle$), 6 ($\circ$), 20 ($\bullet$) and 40 mg/kg ($\triangle$) (C); and terbutaline at oral dose of 30 ($\blacktriangle$), 100 ($\circ$), 300 ($\bullet$) and 1000 mg/kg ($\triangle$) (D). The right panels (E) to (H): the receptor occupancy-time curves after the administration of same dosages as each left side for salbutamol or terbutaline. The receptor occupancy was calculated from the equation as described in the method. Each point represents the mean of 3 - 6 animals.

Photo 1. Focal myocardial fibrosis observed on Day 7 after single treatment of rats with β_2 -AR agonist. The fibrosis of myocardial papillary muscle was caused by intravenous administration of saline (Upper panel; control), and 60 mg/kg salbutamol (Lower panel). The heart was taken by the procedures as described in the method. The region with myocardial fibrosis was stained with Masson-trichrome 1 week after treatment with β_2 -AR agonists ($\times 114$). Fibrous tissue surrounding the small blood vessels and in the intercellular space was stained in all treatment groups.

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mer of both drugs, because their effects are contributed entirely from the (*R*)-enantiomer (Johnson, 1998; Waldeck, 2002). The (*R*)- and (*S*)-enantiomers could be completely separated not only for salbutamol but also for terbutaline according to the method of Bergés *et al.* (1999). The actions on β_2 -AR from the (*R*)-enantiomers of salbutamol and terbutaline were at least 100- and 1000-fold stronger than the respective (*S*)-enantiomer (Buckner and Abel, 1974; Källström *et al.*, 1996); therefore the contribution of the (*S*)-enantiomer as the β -AR stimulant has been shown to be negligible. We also determined the plasma protein binding of both

drugs as the (*R*)-enantiomers, because only unbound fractions are able to bind to the receptor to express the pharmacological effects. Based on the concentration of (*R*)-enantiomer and the plasma protein binding, the receptor occupancies of the drugs were calculated.

The extent of myocardial fibrosis was independent of the $C_{\max}$ and AUC after the administration of both agonists. After conversion of the plasma concentration to receptor occupancy, however, no significant correlation ($r^2 = 0.12$) was observed between the degree of myocardial fibrosis and the AUC Φ without the threshold. When the multiple correlation coefficients were plotted against the threshold (Fig. 4), myocardial fibrosis significantly correlated ($r^2 > 0.78$) with the AUC Φ with the threshold above approximately 50%. The maximum multiple correlation coefficient obtained was approximately 0.9 at the threshold designed 60%. When the threshold was designed at 70%, the multiple correlation coefficient declined because the AUC Φ decreased with increasing threshold.

The receptor occupancy theory has been applied to evaluate the adverse/toxic effect of β -AR antagonists (Yamada *et al.*, 1995a, 1995b) or D₂-receptor antagonists (Yamada *et al.*, 2002). These studies suggest that the receptor-mediated pharmacological effect of drugs depends on receptor occupancy. On the other

Fig. 3. The area of the myocardial fibrosis after treatment with β_2 -agonists was plotted against the AUC Φ ($\% \cdot \text{hr}$) designed A; without the threshold, B; with the occupancy of 50% as the threshold. Each point represents the mean $\pm$ standard error of 3 - 6 animals.

Fig. 4. Relationship between the multiple correlation coefficient and the designated threshold. The area of the myocardial fibrosis after treatment with β_2 -AR agonists was plotted against the AUC Φ ($\% \cdot \text{hr}$) above the variable occupancy designated as the threshold, and the multiple correlation coefficients corresponding to the threshold were obtained. The multiple correlation coefficient was plotted against the threshold.

hand, the present study provides evidence that the chronotropic effect from the β_1 -adrenergic side effects of β_2 -AR agonists causes myocardial fibrosis related to the receptor occupancy-time profile, the AUC Φ with the threshold being $\geq 50\%$, suggesting that AUC Φ is an important indicator for evaluating the side effects of β_2 -AR agonists.

In conclusion, the receptor occupancy theory is useful to predict quantitatively the cardiovascular side effects at earlier stages in the development of β_2 -AR agonists.

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