QUANTITATIVE ESTIMATION OF MYOCARDIAL FIBROSIS BASED ON RECEPTOR OCCUPANCY FOR $\beta_2$-ADRENERGIC RECEPTOR AGONISTS IN RATS

Satoru TANAKA, Yasunori MOMOSE, Masaru TSUTSUI, Tomoyuki KISHIDA, Junji KURODA, Nobuo SHIBATA, Takemi YOSHIDA and Ryoichi YAMAGISHI

Division of Toxicological Research, Kissei Pharmaceutical Co. Ltd.,
2320-1 Ohaza Maki, Hotaka-machi, Minamiazumi-gun, Nagano 399-8305, Japan
Department of Biochemical Toxicology, School of Pharmaceutical Sciences, Showa University,
1-3-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan

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ABSTRACT — To develop $\beta_2$-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 $\beta_1$-ARs in rats as a possible cause for the side effect of $\beta_2$-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 $\beta_2$-AR agonists, at higher dose levels. To evaluate receptor occupancy, plasma concentrations of (R)-salbutamol and (R)-terbutaline, plasma protein binding and the EC$_{50}$ for chronotropic effects in rats were determined. Based on the plasma concentrations, the plasma protein binding and EC$_{50}$, receptor occupancy-time profiles were constructed. The relationship between the receptor occupancy-time profile under the curve, the AUC, 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 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 $\beta_2$-AR agonists.

KEY WORDS: $\beta_2$-adrenergic receptor agonists, Receptor occupancy, Myocardial fibrosis

INTRODUCTION

$\beta_2$-adrenergic receptor (AR)s are subdivided into $\beta_1$-, $\beta_2$- and $\beta_3$-subtypes and are widely distributed in cardiac muscle, adipose tissue, bronchi smooth muscle, gastrointestinal tract, blood vessels and uterus. $\beta_2$-AR is involved in the relaxation of smooth muscle in the bronchi, gastrointestinal tract, blood vessels and uterus, and thus selective $\beta_2$-AR agonists are widely used for the treatment of asthma and premature birth. Salbutamol and terbutaline were the first selective $\beta_2$-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 $\beta$-ARs into $\beta_1$ and $\beta_2$ (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 $\beta_1$-AR which predominates in the cardiac muscle of most experimental animals rather than $\beta_2$-ARs (Vago et al., 1984; Summers et al., 1987). In the initial process for the development of highly selective $\beta_2$-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 $\beta_2$-ARs and/or serotonin receptors (Yamada et al., 1995a).

Correspondence: Satoru TANAKA

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In the present study, in order to assess the myocardial fibrosis produced by \( \beta_2 \)-AR agonists by using receptor occupancy, we evaluated the relationship between the peripheral \( \beta_2 \)-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\(_2\)PO\(_4\) 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 \( \gamma \)-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, 10^4 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 \( \beta_2 \)-AR against the maximum response to isoproterenol, a non-selective \( \beta \)-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\textsuperscript{®} 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-
Myocardial fibrosis by β-adrenergic receptor agonists.

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 \( \beta_1 \)-AR agonism from the \( \beta_2 \)-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-

![Graph](image-url)
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 terbutaline 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 AUCΦ (%·hr) above the variable occupancy designed as the threshold, and multiple correlation coefficients (r^2) corresponding to the threshold were obtained. The relationship between AUCΦ, 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 AUCΦ with the threshold of 50% (Fig. 3B). Fig. 4 shows the relationship between the threshold of AUCΦ and the correlation coefficients. No correlation was observed when the threshold was up to 40% (r^2 ≤ 0.25), whereas correlation was observed when the threshold was ≥ 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 300-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)-enatio-
Myocardial fibrosis by β-adrenergic receptor agonists.

Fig. 2. Plasma concentration- and receptor occupancy of β1-AR - time curves after administration of β2-AR agonists to rats. The left panels: the plasma concentration-time curves after the administration of salbutamol at intravenous dose of 2 (▲), 6 (○), 20 (●) and 60 mg/kg (△) (A); salbutamol at oral dose of 30 (▲), 100 (○), 300 (●) and 1000 mg/kg (△) (B); terbutaline at intravenous dose of 2 (▲), 6 (○), 20 (●) and 40 mg/kg (△) (C); and terbutaline at oral dose of 30 (▲), 100 (○), 300 (●) and 1000 mg/kg (△) (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.
Focal myocardial fibrosis observed on Day 7 after single treatment of rats with β2-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 β2-AR agonists (× 114). Fibrous tissue surrounding the small blood vessels and in the intercellular space was stained in all treatment groups.
Myocardial fibrosis by β-adrenergic receptor agonists.

The myocardial fibrosis was evaluated by measuring the area of fibrosis, which was expressed as a percentage of the area of the myocardium, using a microcomputer system. The area of fibrosis was plotted against the AUCΦ (%)·hr, where AUCΦ is the area under the curve of the plasma concentration of the drug as a function of time. The AUCΦ values were obtained from the plasma concentration-time profiles of the drugs. The AUCΦ values were calculated by the trapezoidal rule, taking into account the amount of drug administered and the duration of treatment.

Fig. 3. The area of the myocardial fibrosis after treatment with β2-agonists was plotted against the AUCΦ (%)·hr. The area of fibrosis was calculated by subtracting the area of the control group from the area of the treatment group. The graph shows the relationship between the AUCΦ (%)·hr and the area of fibrosis. The area of fibrosis was determined by a computer program that automatically calculates the area of the fibrous tissue from the microphotographs.

Fig. 4. Relationship between the multiple correlation coefficient and the threshold. The area of myocardial fibrosis after treatment with β2-AR agonists was plotted against the AUCΦ (%)·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.

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 hand, the area of myocardial fibrosis was plotted against the AUCΦ (%)·hr when the drug is administered at different doses. The area of fibrosis was determined by a computer program that automatically calculates the area of the fibrous tissue from the microphotographs.

Myocardial fibrosis was observed in all groups treated with β2-AR agonists, but the area of fibrosis was smaller in the group treated with the (S)-enantiomer than in the group treated with the (R)-enantiomer. The area of fibrosis was also smaller in the group treated with the (S)-enantiomer than in the group treated with the (R)-enantiomer of the same drug. The area of fibrosis was inversely proportional to the occupancy of the β2-AR receptor. The area of fibrosis was also inversely proportional to the occupancy of the β2-AR receptor when the drug was administered at different doses. The area of fibrosis was determined by a computer program that automatically calculates the area of the fibrous tissue from the microphotographs.

The area of myocardial fibrosis was plotted against the AUCΦ (%)·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.

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hand, the present study provides evidence that the chronotropic effect from the $\beta_1$-adrenergic side effects of $\beta_2$-AR agonists causes myocardial fibrosis related to the receptor occupancy-time profile, the AUCΦ with the threshold being ≥50%, suggesting that AUCΦ is an important indicator for evaluating the side effects of $\beta_2$-AR agonists.

In conclusion, the receptor occupancy theory is useful to predict quantitatively the cardiovascular side effects at earlier stages in the development of $\beta_2$-AR agonists.

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