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Pharmacological Effects of KR60886, A New β_3 Adrenoceptor Agonist

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Abstract – In an attempt to develop new anti-diabetic agents, a series of aryloxypropanolamine derivatives was synthesized to serve as β_3 adrenoceptor agonists. Among these derivatives, 1-{1-methyl-3-[4-(2-methyl-2*H*-1,2,3,4-tetrazol-5-yl)phenyl]propylamino}-3-phenoxy-2-propanol (KR60886) possessed a high affinity for the β_3 adrenoceptor (Ki =28 nM) and moderate affinities for β_1 and β_2 adrenoceptors (Ki =95 nM and 100 nM, respectively). In addition, KR60886 stimulated cAMP production with an EC_{50} of 0.4 μM, confirming its agonistic activity for the β_3 adrenoceptor. *In vivo* activities of KR60886 were examined by using a fat-fed/streptozotocin (STZ)-treated rat model and the ob/ob mouse model. Oral administration of KR60886 (10 mg/kg) for 3 days (*b.i.d.*) to fat-fed/STZ-treated rats significantly lowered plasma glucose levels and reduced plasma free fatty acid concentrations. Similarly, KR60886 treatment (10 mg/kg/day for 7 d) resulted in a reduction of plasma glucose concentrations in ob/ob mice. The present study suggests that KR60886 is a potent β_3 receptor agonist with *in vivo* anti-diabetic properties.

Key words □ β_3 agonist, selectivity, anti-diabetic activity, ob/ob mouse, fat-fed/STZ-treated rat

Since the cloning of human β_3 adrenoceptors from genomic libraries (Emorine *et al.*, 1989), the importance of the β_3 adrenoceptor on human adipocyte function has been extensively investigated (Arch *et al.*, 1984; Lowell and Flier, 1997). However, initial characterization of the receptor has been complicated due to a lack of selective compounds. Consistent with earlier studies reporting atypical β receptor activity, subsequent studies have shown that the β_3 adrenoceptor is expressed mainly in adipose, gastrointestinal and myocardial tissues, and is involved in fat metabolism (Strosberg 1997). Furthermore, selective β_3 adrenoceptor agonists such as BRL-37344 and CL-316,243 were shown to be potent stimulants of lipolysis and thermogenesis, which would lead to an elevation of glucose metabolic rate via an increase of cAMP levels and upregulation of the uncoupling protein UCP1 (Lowell and Flier, 1997; Bloom and Claus, 1994). Therefore, a selective human β_3 adrenoceptor agonist may be a potential therapeutic agent for obesity and type 2 diabetes mellitus (Arch *et al.*, 1984). Unfortunately, the development of β_3 adrenoceptor agonists as therapeutic agents has been hampered by pharmacological differences between rodents and humans (Rosenbaum *et al.*, 1993; Blin *et al.*, 1994; Himms-Hagen and Danforth, 1996). Furthermore, achieving an appropriately high selectivity of β_3 agonists has been difficult, with the implication of possible side effects mediated by β_1 and β_2 adrenoceptors. Recently, substituted oxazole benzenesulfonamides were reported to be potent β_3 agonists exhibiting good selectivity and excellent oral bioavailability in dogs (Ok *et al.*, 2000).

In an effort to develop new anti-diabetic agents, we synthesized and evaluated several aryloxypropanolamine derivatives in relation to their β_3 adrenoceptor agonistic activity as well as for their efficacy as anti-diabetic agents. To do so, we established an experimental model of type 2 diabetes, mimicking the history of human type 2 diabetes, by combining a high fat diet with STZ treatment in rats (Reed *et al.*, 2000). Among the compounds tested, KR60886, an aryloxypropanolamine with a phenyltetrazol substituent (see structure in Figure 1), was selected for further characterization. In the present study, we report on the *in vitro* and *in vivo* efficacies of KR60886. KR60886 was shown to have a good affinity for β_3 adrenoceptors with some selectivity against β_1 and β_2 adrenoceptors. As expected from a functional study looking at cAMP stimulation, oral administration of KR60886 to either fat-fed/STZ-treated rats or to ob/ob mice resulted in a decrease in plasma glucose as well as in plasma free fatty acid concentrations, confirming the anti-dia-

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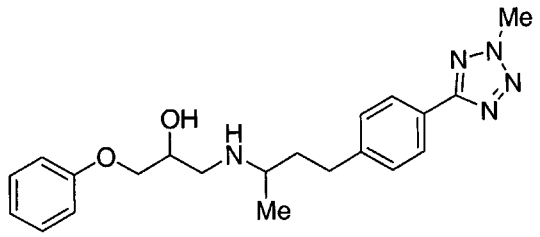

Fig. 1. Chemical structure of KR60886.

betic properties of this compound.

MATERIALS AND METHODS

Materials

Sprague-Dawley rats (5 weeks old) were obtained from Charles River (Atsugi, Japan). Normal chow diet was obtained from Jeilfeed (Taejon, Korea) and the high fat diet was obtained from Harlan Teklad (Adjusted Fat Diet; Madison, WI). C57BL/6J Lep ob/ob mice were bred in facilities at Korea Research Institute of Chemical Technology. Experimental conditions for animal studies conformed with the European Community guidelines for the use of experimental animals. STZ, propranolol, 3-isobutyl-1-methylxanthine (IBMX), phosphocreatine, ATP, EDTA, glucose Trinder and isoproterenol were obtained from Sigma/Aldrich (St Louis, MO). RB-HBETA3 was obtained from Receptor Biology Inc. (Beltsville, MD). β_1, β_2 receptor preparations, [^{125}I]-iodocyanopindolol and [^{3}H]CGP12177 were obtained from NEN (Boston, MA). Creatine phosphokinase was obtained from Boehringer Mannheim (Germany). The cyclic AMP assay kit used here was obtained from Diagnostic Products Corporation (Los Angeles, CA), the free fatty acid assay kit was from Nippon shogi (Japan) and the insulin assay kit was obtained from Linco Research (St. Charles, MO). A cell harvester (CH-5605 Dottikon, Inotech), spectrophotometer (UV-160A, Shimadzu) and liquid scintillation counter (1450 Microbeta, Wallac) were also used in experiments. BRL-37344 was synthesized in our laboratory.

Methods

β adrenoceptor binding assays

To determine the binding affinity of KR60886 for the β_3 adrenoceptor, a receptor binding assay was carried out using human β_3 receptor-expressed cell membranes (RB-HBETA3) on 96-well plates. RB-HBETA3, a human SK-N-MC neuroblastoma cell membrane preparation known to express pharmacologically active β_3 adrenoceptors, was pretreated with 1 μM isoproterenol for 24 hr to remove the limited amounts of β_1 adrenoceptors (Esbenshade *et al.*, 1992; Curran and Fishman, 1996). Membrane (RB-HBETA3, 10 mg) was incubated in assay buffer (50 mM Tris, 12.5 mM $MgCl_2$, 2 mM EDTA, pH 7.4) containing [^{125}I]-iodocyanopindolol (1.4 nM, 2200 Ci/mmol) in the presence or absence of KR60886 (1 nM-1 mM) for 100 min at 37°C in a total volume of 100 μl. Propranolol (1 mM) was used to define non-specific binding and 0.1 μM propranolol to block remaining β_1 receptor binding sites. The incubation mixture was filtered over Filtermat A (Wallac), washed with ice-cold Tris assay buffer 3 times and measured for radioactivity by MicroBeta counter (Wallac 1450 Plus) after Melt-iLex A scintillant treatment. BRL-37344 dissolved in DMSO was studied as a positive control.

The affinities of KR60886 for β_1 and β_2 adrenoceptors were determined using a recombinant receptor preparation expressed in Sf9 cells (30 μg in each well). [^{3}H]CGP12177 (0.9 nM, 30 Ci/mmol) was used as a radiolabeled ligand and incubated for 60 min at 27°C in the presence or absence of KR60886 (1 nM-1 mM). Subsequent procedures followed the same protocols as described above. Ki values were calculated using Prism software (Graphpad Software, Inc.).

cAMP measurement

RB-HBETA3 (10 mg) was incubated with phosphocreatine (22 mM), creatine phosphokinase (1 mg/ml), ATP (0.2 mM), IBMX (0.5 mM), and ascorbic acid (0.02 %) in the presence or absence of KR60886 (10 nM-1 mM) at 30°C for 20 min. EDTA (33 mM) was added to stop the reaction, and then the reaction mixtures were centrifuged and the amount of cAMP produced was measured using a [^{3}H]cAMP assay kit (DPC) according to the manufacturer's instructions. Isoproterenol and BRL-37344 dissolved in DMSO were used as positive controls.

Establishment of fat-fed/STZ-treated animal model

Male Sprague-Dawley rats (5 weeks old) were fed either a normal chow diet (12% of total calories as fat) or a high fat diet (40% of total calories as fat) for 2 weeks. Animals on both diets were then given STZ (40 mg/kg) intravenously under ketamine (65 mg/kg) anesthesia. After 3 days, blood samples (1.5 ml) were collected by heart puncture and analyzed for plasma glucose, insulin, and free fatty acid concentrations after centrifugation of heparin-treated blood samples. Plasma glucose was assayed using a glucose Trinder kit (Sigma) according to the manufacturer's instructions. Plasma free fatty acid was assayed with a Nescauto NEFA kit (Nippon shogi) according to the manufacturer's instructions, while plasma insulin was assayed

by radioimmunoassay (Linco Research, St. Charles, MO).

Anti-hyperglycemic effect of KR60886

To examine the *in vivo* effect of KR60886, fat-fed/STZ-treated rats and ob/ob mice were used. The rats were given KR60886 (10 mg/kg, *b.i.d.*) for 3 days orally, after which plasma glucose, insulin and free fatty acid concentrations were analyzed. Separately, the effect of KR60886 on plasma glucose, insulin and fatty acid concentrations was also examined in the ob/ob mice (9 weeks of age). KR60886 (10 mg/kg) was suspended in 0.5% carboxymethylcellulose (CMC) and administered orally once a day for 7 days. Plasma concentrations of glucose, insulin and fatty acids were measured at day 1, 4 and 7. Rosiglitazone and BRL-37344 in 0.5% CMC were used as reference compounds.

Statistical analysis

Data were expressed as mean±SEM, and statistical significance was assessed by one-way analysis of variance (ANOVA) followed by Dunnetts test.

RESULTS

β adrenoceptor binding assays

Figure 2 shows that KR60886 was capable of displacing [^{125}I]-iodocyanopindolol bound to β_3 adrenoceptors. The Ki value for KR60886 was determined to be 28 nM. Under the same experimental conditions the Ki value for BRL-37344, a known β_3 adrenoceptor agonist, was found to be 1.1 μM (results not shown). To determine the specificity of KR60886 for β_3 adrenoceptors, the affinities of the compound for β_1 and β_2 adrenoceptors were also determined. KR60886 exhibited moderate affinities for β_1 and β_2 receptors, with Ki values of 95 nM and 100 nM, respectively being determined (Figure 2).

cAMP measurement

The *in vitro* functional efficacy of KR60886 to stimulate cAMP production by RB-HBETA3 cell membranes was evaluated. cAMP production was induced in a KR60886 concentration-dependent manner (Figure 3), for which an EC_{50} of 0.4 μM was estimated. As positive controls, isoproterenol, a nonselective β adrenoceptor agonist and BRL-37344 were used. As shown in Figure 3, the intrinsic potency of KR60886 in stimulating cAMP production was about 70% compared with that of isoproterenol. In addition, BRL-37344 also acted as a partial agonist, which is consistent with a previous report (Esbenshade

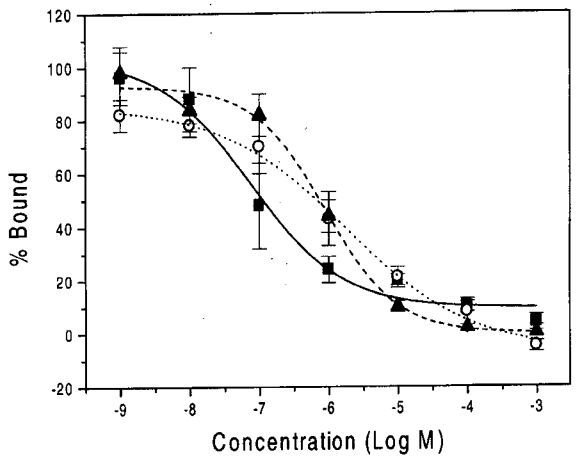

Fig. 2. β adrenoceptor binding profile of KR60886. In the β_3 receptor binding assay, RB-HBETA3 cell membranes were incubated with [^{125}I]- iodocyanopindolol in the presence or absence of KR60886 for 100 min at 37°C. Recombinant β_1 or β_2 receptor preparations were incubated with [^{3}H]CGP12177 in the presence or absence of KR60886 for 60 min at 27°C. The incubation mixture was filtered over Filtermat A, washed, and measured for radioactivity. Values represent mean±SEM of three experiments. Symbols: β_3 (■), β_1 (▲), β_2 (○).

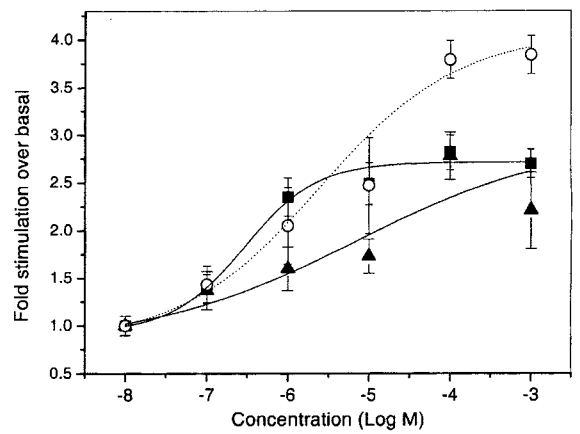

Fig. 3. Stimulation of cAMP production by KR60886. RB-HBETA3 cell membranes were incubated in the presence or absence of KR60886. The cyclic AMP produced was assayed by competition with [^{3}H] cAMP to cAMP binding protein. Values represent mean±SEM of three experiments. Symbols: KR60886 (■), isoproterenol (○), BRL-37344 (▲).

et al., 1992). Based on these results, KR60886 appears to be an agonist for β_3 adrenoceptors.

Anti-hyperglycemic effect of KR60886

The *in vivo* effect of KR60886 was examined using the fat-fed/STZ-treated rat model. It has been reported that a combination of a high-fat diet and STZ treatment of rats simulates a

state that mimics type 2 diabetes states in humans (Reed *et al.*, 2000). In the present study, we prepared fat-fed/STZ-treated rats and measured plasma glucose levels, which were found to reach approximately 650 mg/dl in the absence of any anti-hyperglycemic treatment, which is consistent with previously reported levels (Reed *et al.*, 2000).

Figure 4 shows the anti-hyperglycemic effect of KR60886. Compared with the untreated group, KR60886 treatment reduced plasma glucose concentrations by about 31% (41% normalization) (Figure 4A). KR60886 had no effect on plasma insulin concentrations (Figure 4B), but free fatty acid concentrations were significantly reduced by 19% from untreated group (27% normalization) (Figure 4C). BRL-37344 and also rosiglitazone, a well known insulin sensitizer, showed similar biological effects except that no statistically significant effect on free fatty acid concentration was observed with rosiglitazone treatment. In a similar manner, the *in vivo* glucose lowering effect of KR60886 was also observed in ob/ob mice (Figure 5). In the present study, KR60886 (10 mg/kg) did not affect on plasma insulin and fatty acid concentrations in ob/ob mice.

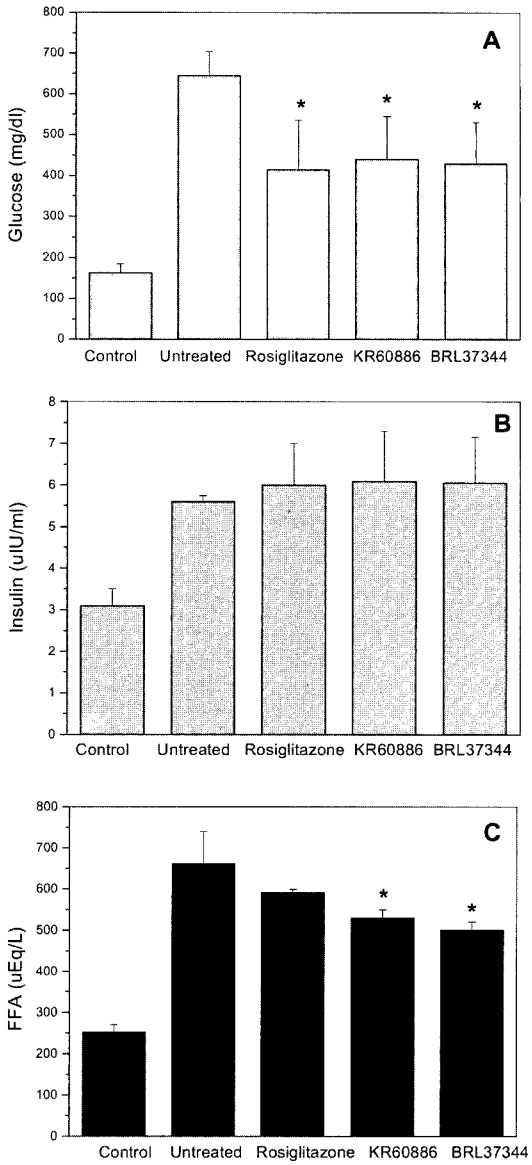

Fig. 4. Effect of KR60886 on plasma glucose (A), insulin (B) and free fatty acid (C) concentrations in fat-fed/STZ-treated rats. Animals were given KR60886 (10 mg/kg) or rosiglitazone (10 mg/kg) or BRL-37344 (10 mg/kg) for 3 days (*b.i.d.*, po). Plasma samples were collected and analyzed for glucose, insulin and free fatty acid concentrations. Control represents non-diabetic SD rats with vehicle treatment (0.5% CMC). Untreated group represents fat-fed/STA-treated rats with vehicle treatment. Values represent mean±SEM of three experiments. (each experiment, N=5, *P<0.05 vs untreated group).

DISCUSSION

The β_3 adrenoceptor was first described in rodent adipose tissue (Arch *et al.*, 1984) and its pharmacological profile was subsequently characterized (Emorine *et al.*, 1989). Similar to β_1 and β_2 receptors, the β_3 receptor is a Gs protein-coupled receptor with 7 transmembrane domains. β_3 adrenoceptor-induced lipolysis is known to be mediated by cAMP elevation followed by lipoprotein lipase activation (Lowell and Flier, 1997). In addition, PKA activation by cAMP increases the transcription of uncoupling protein (UCP). UCP subsequently induces uncoupling of the electron transport chain in mitochondria, producing changes in glucose metabolic rate (Arch and Kaumann, 1993). Since β_3 adrenoceptor activation induces lipolysis and has a blood glucose lowering effect, various β_3 adrenoceptor agonists have been synthesized in an effort to develop novel anti-diabetic agents (Weber, 1998; Weyer *et al.*, 1999). Previously, several compounds such as pyridylethanolamine analogues and substituted oxazole benzenesulfonamides were reported as potent human β_3 adrenoceptor agonists (Ok *et al.*, 2000; Mathvink *et al.*, 2000). In the present investigation, we synthesized aryloxypropanolamine derivatives based on modification of the endogenous ligands, epinephrine and norepinephrine. From β_3 receptor binding assays, an aryloxypropanolamine derivative with a phenyltetrazol substituent, KR60886 was chosen for *in vivo* screening since, in preliminary experiments, it displayed a high affinity for the β_3 receptor.

To determine the binding affinity for β_3 receptors, we employed RB-HBETA3 preparations which express human β_3 receptors and possibly some β_1 receptor sites at a much lower level (Curran and Fishman, 1996). Since we used 0.1 μM propranolol to

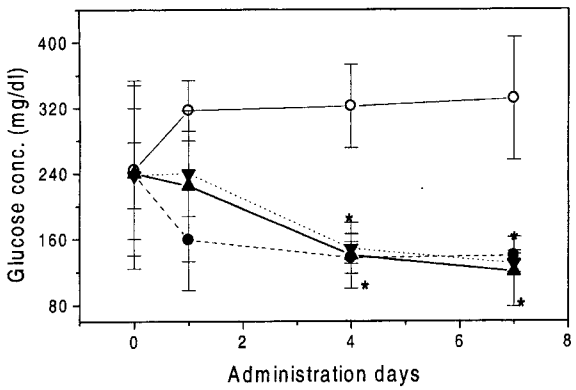

Fig. 5. Effect of KR60886 on plasma glucose in ob/ob mice. KR60886 (10 mg/kg) or rosiglitazone (10 mg/kg) or BRL-37344 (10 mg/kg) was administered orally for 7 days (once a day). Plasma glucose concentrations were determined at day 1, 4 and 7. Data represent mean±SEM (N=5). Symbols: Control (○), Rosiglitazone (●), KR60886 (▼), BRL-37344 (▲). *P<0.05 vs control.

block remaining β_1 binding sites, the affinity of KR60886 reported in this study (Ki=28 nM) is likely to be on the β_3 receptors. As a reference compound, BRL-37344, with an estimated Ki value of 1.1 μM, was used. Given that this Ki is similar to that reported previously for this compound (Emorine *et al.*, 1994), KR60886 appears to have a 39-fold higher affinity for the β_3 adrenoceptor than does BRL-37344.

The agonistic activity of KR60886 on β_3 adrenoceptors is best described by its ability to stimulate adenylyl cyclase in a functional assay. KR60886 increased cAMP production in a concentration-dependent manner in HBETA3 cell membranes, indicating that it acts on β_3 receptors as a high affinity agonist. However, the intrinsic efficacy of KR60886 was only about 70% that of isoproterenol, indicating that KR60886 may not be a full agonist. On the other hand, while the maximum fold stimulation of HBETA3 cell membranes by isoproterenol was similar to the value reported previously (Guan *et al.*, 1995), the EC_{50} value in the present study was higher than the previously reported value. This discrepancy is likely due to the differences between intact cell preparations and broken cell membrane preparations. Although we cannot rule out the possibility that KR60886 may stimulate cAMP production via remaining β_1 sites, it is highly likely that the compound acts as a β_3 agonist since we used 0.1 μM propranolol to block the β_1 sites. In addition, KR60886 showed lipolytic activity in 3T3-F442 cells to a similar degree as BRL37344, further suggesting the β_3 agonistic property of the compound.

Using fat-fed/STZ-treated rats and ob/ob mice, KR60886 was shown to have *in vivo* glucose lowering effects. In addition, plasma free fatty acid levels were also reduced by KR60886 treatment. These results are consistent with the observation that KR60886 induces lipolysis in 3T3-F442 cells (unpublished results). Since selective β_3 adrenoceptor antagonists are not available, it was not possible to examine if the *in vivo* effect of KR60886 could be reversed by such an antagonist. Based on the moderate affinities of KR60886 for β_1 and β_2 adrenoceptors, it is possible that KR608886 may interact with β_1 and β_2 receptors when administered *in vivo*, subsequently inducing related side effects. However, the *in vivo* glucose lowering efficacy of KR60886 is probably mediated by β_3 adrenoceptor binding because BRL-37344, which is well-known as a β_3 adrenoceptor agonist, displayed a very similar profile to that of KR60886. In addition, it is possible that the plasma glucose-lowering effect may be mediated only by the β_3 receptor among the adrenoceptor subtypes. Although the binding affinity of BRL-37344 is lower than that of KR60886, the *in vivo* efficacy of BRL-37344 is somewhat better than that of KR60886, one possible reason being due to their different pharmacokinetic profiles.

In summary, we have identified KR60886, an aryloxypropanolamine derivative, as a high affinity β_3 adrenoceptor agonist. As expected from its mechanism of action, KR60886 exhibited *in vivo* plasma glucose lowering effects in two different diabetic animal models. The findings reported here allude to the fact that KR60886 may be a good starting point in the development of a novel anti-diabetic agent. Improvements in anti-hyperglycemic efficacy and selectivity should be accomplished by further structural modifications of aryloxypropanolamine derivatives.

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