Anti-Obesity Effects of Selective Agonists to the β3-Adrenergic Receptor in Dogs. I. The Presence of Canine β3-Adrenergic Receptor and *in vivo* Lipomobilization by Its Agonists

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ABSTRACT. It is known that in rodents and humans the β3-adrenergic receptor (β3-AR) is present primarily in adipocytes and plays a significant role in the adrenergic stimulation of lipolysis. We examined the expression of β3-AR mRNA in the dog and the lipomobilizing effects of β3-AR-selective agonists *in vivo*. Reverse transcription polymerase chain reaction of RNA extracted from dog adipose tissue produced a cDNA fragment, the nucleotide sequence of which was highly homologous to the corresponding regions of human (86.4%) and mouse (79.5%) β3-AR cDNA. The β3-AR mRNA was present at high levels in subcutaneous and visceral adipose tissues, but undetectable in other organs. When a selective β3-AR agonist, CL316,243, was infused intravenously into beagle dogs, the plasma level of free fatty acid increased in 30 min and persisted at higher levels for several hours. ICI D7114, another β3-AR agonist, also showed a similar lipomobilizing effect, but with lower potency. β3-AR agonist infusion also increased the plasma insulin level. These results suggested that functional β3-AR is present in adipose tissues of the dog and that it is effective for *in vivo* lipomobilization. — KEY WORDS: adipose tissue, adrenergic agonist, β3-adrenergic receptor, canine, lipomobilization.

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In mammals, there are two types of adipose tissues, white and brown adipose tissues. Although both tissues consist of a large number of adipocytes accumulating triglycerides, their physiological roles contrast: that is, white adipose tissue is the major site of energy storage and releases fatty acids into blood, while brown adipose tissue oxidizes fatty acids to produce heat and is the site of energy expenditure. Hydrolysis of triglycerides in adipocytes is largely dependent on the β-adrenergic action of catecholamines. Pharmacological and biochemical studies have demonstrated that three isoforms of the β-adrenergic receptor (AR), β1-, β2-, and β3-ARs, coexist in white and brown adipocytes [8, 13, 14]. β3-AR is expressed primarily in adipocytes [12], whereas β1- and β2-ARs are present in a wide variety of cell types. Thus, it seems rational to expect that agonists to β3-AR might be selective stimulants of white fat lipolysis and brown fat thermogenesis, and thereby be useful as anti-obesity drugs. In fact, the recent cDNA cloning of β3-AR from human [6], mouse [18] and rat [9, 16] sources has promoted the development of various β3-AR agonists.

We have examined the effects of several β3-AR agonists on white and brown adipose tissues in a genetically obese, diabetic animal model, yellow KK mice, confirming that chronic administration of the agonists produces hypertrophy of brown adipose tissue, increases expression of a thermogenic protein (uncoupling protein), improves glucose tolerance, and reduces adiposity [17, 20]. Similar anti-obesity and anti-diabetic effects of β3-AR agonists were also reported in other obese mouse and rat models [1, 4, 10]. In veterinary practice for companion animals, obesity is the most common nutritional disorder, as in humans [5, 15]. However, in contrast to rodents and humans, there have been few reports about β3-AR and effects of its agonists in the canine and feline. As a series of experiments for possible application of β3-AR agonists to the pharmacological treatment of companion animal obesity, in this study, we confirmed the presence of canine β3-AR by analysis of its cDNA, and examined the acute lipomobilizing effects of two selective β3-AR agonists in non-obese healthy dogs.

MATERIALS AND METHODS

Animals: One male and six female adult beagle dogs weighing 10–13 kg were used several times each under various experimental conditions. Each experiment was carried out at least 7 days after the last one. The animal care and procedures were in accordance with the guidelines of the Animal Care and Use Committee of Hokkaido University.

Chemicals: A highly selective β3-AR agonist, CL316,243, disodium (R,R)-5 [2-{3-chlorophenyl}-2-hydroxyethyl]-aminopropyl)-1,3-benzodioxole-2,2-dicarboxylate, was provided by American Cyanamid Co. (Pearl River, NY, U.S.A.) [2]. Another β3-AR agonist, ICI D7114 (its acid metabolite), [(S)-4-(2-hydroxy-3-phenoxypropylamino-ethoxy)-N-(2-methoxyethyl)phenoxyacetamide] [11], was

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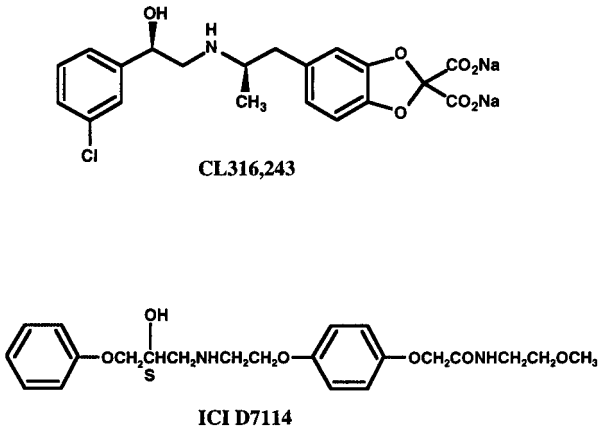

Fig. 1. Chemical structures of the selective β3-adrenergic agonists, CL316,243 and ICI D7114.

provided by ICI Pharmaceuticals (Macclesfield, UK), and a non-selective β-AR agonist, (-)-isoproterenol, by Sigma (St. Louis, MO, USA). Figure 1 shows the chemical structures of these compounds. All drugs were dissolved in sterilized saline solution immediately before use.

RNA extraction and partial sequence of canine β3-AR cDNA: The beagles were deeply anesthetized by pentobarbital sodium (Nembutal; Dinabot, Osaka) injection, and various tissues were obtained and frozen in liquid nitrogen. Total RNA was extracted using TRIzol (Gibco BRL,Tokyo) and its concentration was determined from the absorbance at 260 nm. Total RNA extracted from perirenal adipose tissue was used for reverse transcription polymerase chain reaction (RT-PCR) and subsequent sequencing of the resulting cDNA fragment of canine β3-AR. For RT-PCR, two primers 5'-ATGGCTCCGTGGCCTCAC-3' (forward) and 5'-CCCAACGGCCAGTGGCCAGTCAGCG-3' (reverse) were designed based on the published sequences of murine [18] and human β3-AR cDNAs [6]. Two μg of total RNA was reverse-transcribed at 37°C for 1 hr in 20 μl of 1x first-strandbuffer (Gibco BRL) containing 200 U of M-MLV reverse transcriptase (Gibco BRL), 100 pmoles of oligo$(dT)_{15}$, 0.5 mM dNTP and 10 U of RNase inhibitor (Wako, Tokyo). PCR amplification was performed for 35 cycles at 95°C for 30sec, 60°C for 30sec, and 72°C for 2 min in 50 μl of 1x PCR buffer (Perkin-Elmer, Branchburg, NJ, U.S.A.) containing 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer), 2 mM $MgCl_2$, 200 μM dNTP, 5% DMSO and 1 μM of each primer. The PCR products were ligated with the pCRII vector (Invitrogen, Carlsbad, CA, U.S.A.) and sequenced by the Taq Dye Deoxy Terminator Cycle Sequencing method using an automatic DNA sequencer (Perkin Elmer-Applied Biosystems, model 373A).

In vivo lipomobilizing effect of β3-AR agonist: All experiments were carried out in conscious dogs without any anesthetics and sedatives. After overnight fasting, a catheter for drug infusion was placed into a cephalic vein. For determination of the baseline value, saline solution was infused continuously at 0.6–0.7 ml/min for 30 min, and then the solution containing various drugs was infused at the same rate for 30 min. Blood samples were collected from the jugular vein for 150 min at 15–30 min intervals before, during and after the infusions. Blood samples were centrifuged and the resulting plasma was stored at −20°C.

The plasma concentrations of free fatty acid (FFA) and glucose were determined using commercial assay kits, the NEFA C-test Wako (Wako) and Glucose B-test Wako (Wako), respectively. Plasma insulin level was determined with a commercial kit (Insulin RIA-beads II; Dinabot, Tokyo) using human insulin as a standard.

Data analysis: All values are given as mean ± SE. Statistical analysis was performed by analysis of variance (ANOVA) with a post hoc comparison using Fisher's LSD and the paired *t* test was used for analysis of the differences from time 0.

RESULTS

Nucleotide sequence of a cDNA fragment of canine β3-AR: To confirm the presence of canine β3-AR, total RNA extracted from perirenal white adipose tissue of beagles was subjected to RT-PCR and the resulting PCR fragment of 317 bp was sequenced. As shown in Fig. 2, the nucleotide sequence of the cDNA fragment was 79.5–86.4% homologous to the corresponding region of β3-AR cDNA of other species so far reported [6, 9, 16, 18]. The deduced amino acid sequence was also highly homologous (81.7–82.9%) among these species (data not shown). It is impossible at present to compare the sequence with those of the canine β1- and β2-ARs, because these β-ARs have not been cloned from this species. However, considering the relatively low sequence homology (about 50%) among the β1-, β2- and β3-ARs in other species [6, 16], it is likely that the cDNA fragment obtained in the present study was for canine β3-AR.

Tissue distribution of canine β3-AR: To examine the tissue distribution of canine β3-AR, total RNA extracted from various tissues of adult beagles was analyzed by RT-PCR. As shown in Fig. 3, a clear band of 317 bp was found in every adipose tissue obtained from omental, mesenteric, retroperitoneal and perirenal regions, but not in other tissues. In liver, kidney and skeletal muscle, a smaller band of about 240 bp was observed. This band might not have derived from β3-AR mRNA, because the β3-AR cDNA fragment did not hybridize with it (data not shown).

In vivo lipomobilizing effects of β3-AR agonists: The lipomobilizing actions of two β3-AR agonists, CL316,243 and ICI D7114, were examined *in vivo* by monitoring the plasma FFA response to intravenous infusion of the agonists compared with those of a non-selective β-AR agonist (isoproterenol). Figure. 4 shows the effects of equimolar doses (0.2 nmol/kg/min) of the three agonists on plasma FFA levels in conscious fasting dogs. At this dose, isoproterenol and CL316,243 produced considerable rises in the plasma FFA level, whereas ICI D7114 was without significant effects. The effects of isoproterenol and

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Dog      1: ATGGCTCCGTGGCCTCAC-GGGAACGGCTCTGTGGCCTCGTGGCCGG--CTGCCCCCACC
Human    1: ATGGCTCCGTGGCCTCAC-GAGAACAGCTCTCTTGCCCCATGGCCGGACCT--CCCCACC
Mouse    1: ATGGCTCCGTGGCCTCACAG-AAACGGCTCTCTGGCTTTGTGGTCGGA-C-GCCCCTACC
            ****************** *  *** ***** * **    *** ***  *   *** ***

Dog     61: CCGACG-CCCGA-TGCCGCCAACACGAGTGGGCTGCCAGGGGCGCCCTGGGCGGTGGCCT
Human   61: CTGGCG-CCCAA-TACCGCCAACACCAGTGGGCTGCCAGGGGTTCCGTGGGAGGCGGCCC
Mouse   61: CTG--GACCCCAGTGCAGCCAACACCAGTGGGTTGCCAGGAGTACCATGGGCAGCGGCAT
            * *  * *** * * * ******** ****** ******* *  ** ****  * ***

Dog    121: TGGCGGGGGGCGCTGTTGGCGCTGGAGGTGCTGGCCACCGTGGGAGGCAACCTGCTGGTCA
Human  121: TAGCCGGGGGCCCTGCTGGCGCTGGCGGTGCTGGCCACCGTGGGAGGCAACCTGCTGGTCA
Mouse  121: TGGCTGGGGC-AT--T---GCT---GGCGCTGGCCACGGTGGGAGGCAACCTGCTGGTAA
            * ** *****  *  *   ***   ** ********* ******************** *

Dog    181: TCGTGGCCATCG-CTCGGACGCCAAGACTGCAGACCATGACCAACGTGTTCGTGACGTCG
Human  181: TCGTGGCCATCGCCT-GGACTCCGAGACTCCAGACCATGACCAACGTGTTCGTGACTTCG
Mouse  181: TCATAGCCATCGCC-CGCACGCCGAGACTACAGACCATAACCAACGTGTTCGTGACTTCA
            ** * ******* *  * ** ** ***** ******** ***************** **

Dog    241: CTGGCCACCGCGGACCTGGT-GGTGGGGCTCCTGGTAGTGCCGCC-GGGGGCCACCTTGG
Human  241: CTGGCCGCAGCCGACCTGGT-GATGGGACTCCTGGTGGTGCCGCC-GGCGGCCACCTTGG
Mouse  241: CTGGCCGCAGCTGACTTGGTAG-TGGGACTCCTCGTAATGCCACCAGG-GGCCACATTGG
            ****** * ** *** **** * **** ***** **  **** ** ** ****** ****

Dog    301: CGCTGACGGGCCGCTGGCC-TCTGGG
Human  301: CGCTGACTGGCCACTGGCCGT-TGGG
Mouse  301: CGCTGACTGGCCATTGGCCCT-TGGG
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Fig. 2. Partial nucleotide sequence of the canine β3-AR cDNA. The nucleotide sequence of a cDNA fragment obtained by RT-PCR of total RNA from dog adipose tissue is aligned with those of human and mouse β3-AR cDNAs.

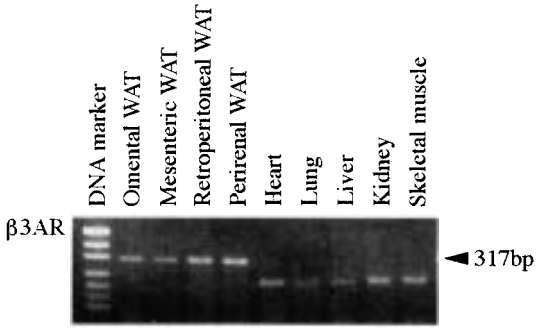

Fig. 3. RT-PCR analysis of β3-AR mRNAs of various dog tissues.

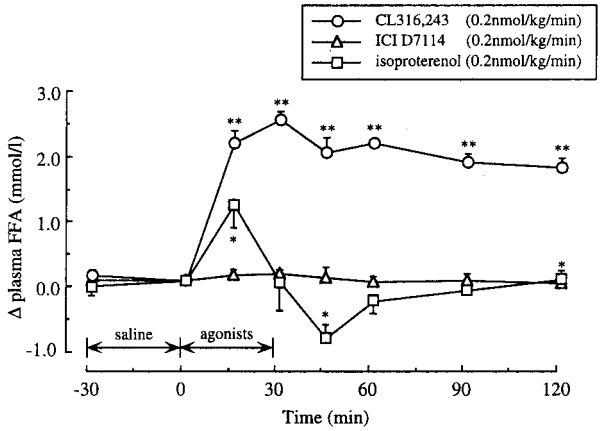

Fig. 4. Effects of intravenous injection of selective (CL316,243, ICI D7114) and non-selective (isoproterenol) β-AR agonists on plasma FFA. Values are means ± SE for 4 experiments and expressed as the changes from those at 0 min. *P<0.05 and **P<0.01, compared with the 0-time values (0.87 ± 0.1 mmol/*l*).

CL316,243 were rapid and the plasma FFA level increased to the maximal or nearly maximal level in 15 min. The effect of isoproterenol was transient and the plasma FFA level returned to a lower level at the end of infusion and thereafter, whereas that of CL316,243 was long-lasting, maintaining a higher plasma FFA level for at least 120 min. Dose-response effects of CL316,243 and ICI D7114 were also examined. As shown in Fig. 5, CL316,243 infusion at 2 nmol/kg/min produced almost the same, but slightly longer-lasting plasma FFA response as at 0.2 nmol/kg/min, but at 0.02 nmol/kg/min the response was reduced. Although no significant effects was found with 0.2 nmol/kg ICI D7114, a higher dose of 2 nmol/kg/min produced a significant plasma FFA response, which was almost the same as that seen with 0.02 nmol/kg/min CL316,243. Thus, both CL316,243 and ICI D7114 were capable of increasing the plasma FFA level in dogs, although the effective doses were about two orders of magnitude different.

Since the plasma FFA level is known to be strongly influenced by insulin in addition to catecholamine, effects of the β3-AR agonists on plasma insulin and glucose were also examined. As shown in Fig. 6, CL316,243 infusion

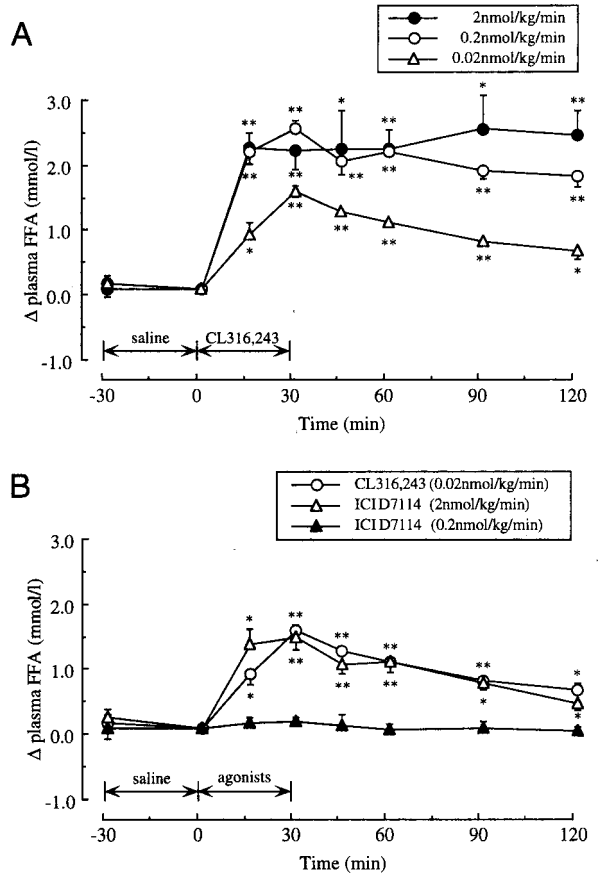

Fig. 5. Dose-response effects of CL316,243 (A) and ICI D7114 (B) on plasma FFA. *P<0.05 and **P<0.01, compared with the 0-time values (1.0 ± 0.1 mmol/*l*).

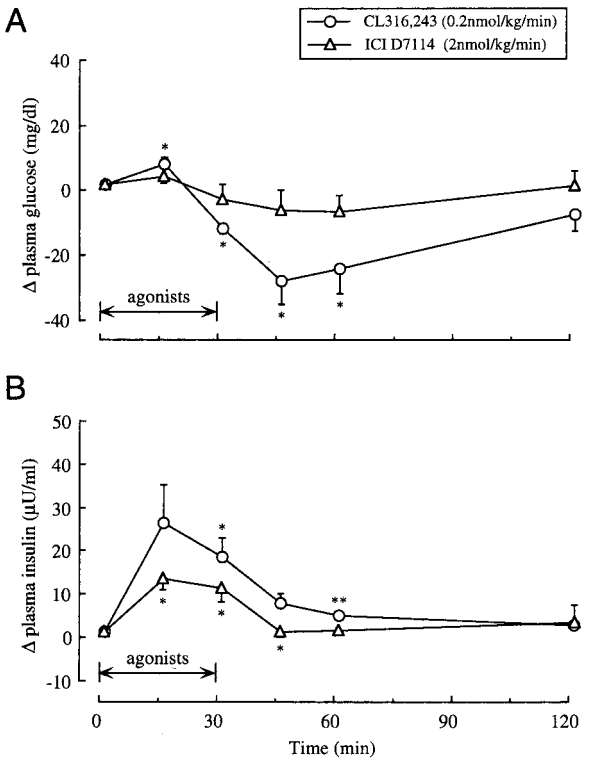

Fig. 6. Effects of CL316,243 and ICI D7114 on plasma glucose (A) and insulin (B) levels. *P<0.05 and **P<0.01, compared with the 0-time values (108.1 ± 4.4 mg/d*l* and 4.6 ± 0.7 μU/m*l*, respectively).

elicited a rapid and significant increase in the plasma insulin level, followed by a gradual decrease of the plasma glucose level. Similar but smaller responses were also found after ICI D7114 infusion.

DISCUSSION

The presence of an atypical β-AR (β3-AR), different from the classical β1- and β2-AR subtypes, was initially proposed in pharmacological studies of lipolysis in adipocytes of rodents [1], and confirmed by cloning of its cDNA in the mouse [18], rat [9, 16], and human [6]. The mouse β3-AR, for example, is a 7-transmembrane helix-type protein composed of 388 amino acids, whose sequence is about 80% identical to those of the rat and human β3-ARs [16, 18]. However, the homologies of the amino acid sequences among β1-, β2- and β3-ARs are rather low (40–50%) in all these species [6, 16]. In the present study, we applied RT-PCR to total RNA of dog adipose tissue using a primer set designed based on mouse β3-AR cDNA, and obtained a cDNA fragment of 317 bp, whose nucleotide sequence was 86.4% homologous to the corresponding region of human β3-AR cDNA. Although the compared region was only about one-fourth of the predicted coding region, the high homology of the nucleotide sequence suggested that the obtained cDNA fragment was amplified from mRNA of canine β3-AR. Pharmacological and molecular biological studies have confirmed that rodent β3-AR is present specifically in adipocytes of the mouse and rat [9, 16]. In the present study, the PCR product of 317 bp was found only in adipose tissues in the dog. These results again support the presence of canine β3-AR and its adipose-specific distribution.

Various compounds having agonistic activity to β3-AR have been developed, mainly using isolated adipocytes and Chinese hamster ovary cells transfected with mouse β3-ARs [2, 4, 11]. In the present study, we used two agonists, CL316,243 and ICI D7114, both of which were confirmed to be selective agonists of rodent β3-ARs [2, 11]. ICI D7114 is an aryloxypropanolamine compound, while CL316,243 is a phenylethanolamine (Fig. 1). Phenylethanolamines such as adrenaline and isoproterenol can generally stimulate all isoforms of β-AR, whereas CL316,243 is highly selective for β3-AR (β3:β2:β1 = 100,000:1:0) [2]. Although the lipolytic activity of the agonists so far developed is known to be quite low for adipocytes of the guinea pig [3] and human [14], some of them are reported to be effective in the dog; that is, they stimulate lipolysis in isolated adipocytes and increase plasma FFA *in vivo* [7]. In the present study, intravenous infusion of CL316,243 and ICI

D7114 into conscious beagls increased the plasma FFA level, suggesting an *in vivo* lipomobilizing action of β3-AR agonists in the dog as well as in rodents. It seems possible that the increased plasma FFA level may be secondary to some changes in the plasma insulin level, which has an anti-lipolytic effect. However, this is unlikely because the β3-AR agonists increased, rather than decreased, the plasma insulin level. Thus, the lipomobilizing effect of the agonists may be due to a direct action on the β3-AR in adipocytes. This is well consistent with the *in vitro* lipolytic effect of other β3-AR agonists in dog adipocytes [8].

In contrast to the transient lipomobilizing action of isoproterenol, the actions of CL316,243 and ICI D7114 were long-lasting, keeping plasma FFA at higher levels for several hours. This may be attributable to slow clearance of these drugs, which is suitable for their *in vivo* pharmacological use. The lipomobilizing potencies of the two compounds were different, that of CL316,243 being higher than ICI D7114. A similar difference was also reported between other β3-AR agonists [7, 8]: BRL37344 (phenylethanolamine) >CGP12177 (aryloxypropanolamine). These results suggest that phenylethanolamines are more effective agonists to β3-AR, at least in the dog, and may be more useful as anti-obesity drugs. The anti-obesity effect of CL316,243 will be reported in the following paper [19].

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