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Effects of clenbuterol enantiomers on growth of young male rats

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Abstract

Clenbuterol (CB) is one of the β2-adrenergic receptor agonists with powerful muscle anabolic and lipolytic effects, and is prohibited as a doping drug for athletes. However, it is one of the candidate countermeasures for aging-related diseases. Previously we reported that CB induced muscular hypertrophy, but inhibited the longitudinal growth of bones in young male rats. However, the mechanism of the inhibitory effect on bone growth is not yet clear. CB is manufactured as a 1:1 racemic mixture of 2 isomers of (-)-R and (+)-S enantiomers, and only the (-)-R enantiomer may have pharmacological activity. We examined the effects of two CB enantiomers, (+)-S-CB and (-)-R-CB, on growth of striated muscle and bone in young male rats. Eighteen male Sprague-Dawley rats (8-wk-old) were randomly assigned to a control (CONT, n = 6) and two CB enantiomers groups ((+)-S-CLEB: n=6, (-)-R-CLEB: n=6). Each CB enantiomer of 2 mg/kg body weight was daily administered subcutaneously for 2 weeks. After treatment, heart and the slow-twitch soleus (SOL) and fast-twitch extensor digitorum longus (EDL) muscles and bones were analyzed. The muscle wet weights of SOL and EDL muscles significantly increased in (+)-S-CLEB (HEART: +28%, SOL: +25%, EDL: +28%) and (-)-R-CLEB (HEART: +27%, SOL: +29%, EDL: +35%). Both (+)-S-CB and (-)-R-CB induced striated muscle hypertrophy (heart, SOL, and EDL). Concerning bones, (+)-S-CB induced decreased tibia length (-1.2%) and decreased femur BMD (-5.8%), and (-)-R-CB induced decreased femur BMD (-8.2%). These results show that (+)-S-CB and (-)-R-CB might work differently at times.

Keywords: β2-adrenergic agonists, clenbuterol enantiomer, striated muscle, femur, tibia

Introduction

It is well known that Clenbuterol (4-amino-α-[[(tert-butylamino) methyl]-3, 5- dichlorobenzyl alcohol) is one of the famous long-acting β2 adrenergic receptor agonists for the treatment of asthma with powerful muscle anabolic and lipolytic effects. In sports as well as in cattle feeding, clenbuterol (CB) is illicitly misused due to its anabolic properties to promote muscle growth1). Therefore, it is prohibited to use as a doping drug for athletes2). We previously showed that it induces muscle hypertrophy, but inhibits longitudinal growth of bones in young male rats, and attention needs to be paid to high dose usage in youth3). However, it is also a pharmacologically interesting candidate as a countermeasure for sarcopenia4) and CNS disease5,6) with aging.

CB is manufactured as a 1:1 racemic mixture (rac-CB) of two enantiomers, (-)-R and (+)-S isomers, and it is generally believed that only the (-)-R enantiomer shows pharmacological activity. It is important to make clear the different actions of enantiomers as seen in the case of the Thalidomide Tragedy7). For the first time, von Deutsch et al.8) reported that both enantiomers had equal anabolic activity in skeletal muscle of mature male rats, but was less active in cardiac muscle hypertrophy than rac-CB. Furthermore, they reported that treatment with 1.0 mg/kg rac-CB significantly increased tibia mass, but had no effect on the femur. In contrast, treatment with 0.4 mg/kg rac-CB or either enantiomer (0.2 mg/kg each) had no effect on bone mass. By the way, in general, it is believed that bone mass is associated with muscle strength related to increased muscle mass, which is inducible with some β2-agonists. Some investigators showed that a powerful β2-agonist reduced BMD loss in denervation9), tail-suspension10) and immobilization11). On the other hand, not only our report of CB, but also Bonnet et al.12,13) showed that salbutamol induced a deleterious effect on bone mass. Takeda et al.14) showed that isoproterenol, a non-specific β-receptor antagonist, decreased bone mass in mice. Togari15) and Arai et al.16) also reported that β2-receptors are expressed in osteoblasts and osteoclasts. It suggests that β2-agonists may act directly on bone and via such effects on muscle. Recently, it was thought that bone remodeling is controlled by the central and peripheral sympathetic ner-
Materials and Methods

Materials and equipment. Both (+)-S-CB and (-)-R-CB were gifts from Pr. Miyamoto (Dept. of Hospital Pharmacy, Kanazawa University). All other chemicals used were of reagent grade. Puriﬁcations were measured with an HPLC system (Jasco, Tokyo, Japan) which was equipped with a pump (PU-2080 Plus, Jasco, Tokyo), a UV/VIS detector (UV-2075 Plus, Jasco, Tokyo), a column oven (CO-2060 Plus, Jasco, Tokyo), and a column (CHIRALCEL OJ-H, Daicel Chemical Industries, Tokyo). The mobile phase consisted of Hexen:Ethanol = 95:5, the flow rate was 1.0 ml/min, the temperature was 30°C, and the detection wavelength (UV) was set at 254 nm. The optical rotations of these compounds were analyzed by DIP-370 Polarimeter (Jasco, Tokyo) which was equipped with a Na lamp and cell length of 50 mm. Both samples of (-)-R- and (+)-S-CB made soluble with 10 mg/ml water and were analyzed.

Animal care and drug treatment. Eighteen male Sprague-Dawley rats (7-wk-old, 200-225 g) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). Fundamentally, animal care was the same as our previous report (2). They were fed standard chow (MF, Oriental Yeast Co. Ltd., Tokyo) and water ad libitum, and were maintained in a 12 hour light-dark cycle, at 25 ± 3°C, with 55 ± 5% humidity. Rats were weighed daily. After a 1-week acclimation period, animals were randomly divided into 3 groups, vehicle control (CONT, n = 6), and 2 CB enantiomers groups ((+)-S-CLEB: n = 6, (-)-R-CLEB: n = 6). Both CB enantiomers of 2 mg/kg body weight were administered once a day subcutaneously for 2 weeks. The control rats were also injected daily with 0.5 ml/kg normal saline solution. The experimental protocol was approved by the Kanazawa University Animal Care and Use Committee. After treatment for 2 weeks, all rats were weighed, anaesthetized and sacriﬁced with an intraperitoneal injection of 50 mg/kg body weight pentobarbital sodium and decapsulation.

Muscle and bone treatments. The soleus (SOL), extensor digitorum longus (EDL), and ventricle (HEART) muscles were quickly removed and weighed. In skeletal muscles, the average values of the right and left were used for data analyses. The ratio of muscle wet weight to body weight was calculated for the calibrated evaluation of animal organs which are body size dependent.

The right and left bones of both femurs (FE) and tibiae (TI) were excised and cleared of fat and connective tissue. The longitudinal lengths of the bones were measured with a stainless steel Vernier caliper (Mitutoyo, Kawasaki, Japan). The BMDs of FE and TI were measured by dual-energy x-ray absorptiometry (DXA) using the Aloka DCS-600R for small animals (Aloka, Tokyo, Japan) with Small Subject software (version 8.5, Aloka). Scanning speed was 10 mm/s, with resolution set at 0.2 x 1 mm. Coefficients of variation were determined from five repeat scans on two tibiae over several days, with repositioning for each scan. The average coefﬁcient of variation was 2.9% for BMD. The minimum limits of measure for this apparatus were 22 mg/cm² for BMD. Data from the thin ﬁbula, which is continual to the TI, was negligible (<1%), but deleted from the data of TI with the software. The average values of right and left bones were used for analyses in this investigation.

Results

Separation of clenbuterol enantiomers with HPLC and Polarimeter. The chiral separation of CB enantiomers was accomplished by HPLC using a CHIRALCEL OJ-H column (15 x 0.46 cm i.d. with 5 μm particles). Fig. 1 shows that (-)-R-CB was eluted at a retention time of 6.742 min (peak area percentage: 99.7%), but the (+)-S-CB was eluted at 7.983 min (peak area percentage: 98.0%) with a little contamination of (-)-R-CB (peak area percentage: 2.0%). Therefore, (-)-R-CB contaminated with (+)-S-CB was estimated as 0.006 mg/kg, and (+)-S-CB contaminated with (-)-R-CB was 0.04 mg/kg. Each optical rotation was (+)-S-CB: 0.160 and (-)-R-CB: -0.184, respectively.

Body weight and muscle weight. Body weight of control rats (CONT) increased normally during the experimental period, but (+)-S-CLEB showed a quick signiﬁcant decrease in weight (P < 0.05) before recovery. By the way, (-)-R-CLEB also showed a rapid signiﬁcant decrease in weight (P < 0.01) for 1-5 days after injection of the drug followed by a gradual recovery. Two weeks later, (+)-S-CLEB and (-)-R-CLEB showed the same body weight.
Fig. 1 Chiral separation of clenbuterol enantiomers by HPLC.

Chromatograms of left (A) are obtained from (-)-R-clenbuterol and right (B) from (+)-S-clenbuterol. The data are obtained using CHIRALCEL OJ-H column (mobile phase: hexane/ethanol = 95:5, flow rate: 1.0 mL/min, UV detection: 254 nm), and details of HPLC analysis are explained in the text.

with CONT, though (+)-S-CLEB showed inhibited growth (-3%, Fig. 2). The body weight and muscle wet weight after CB treatment for 2 weeks are shown in Table 1. Finally, (+)-S-CLEB and (-)-R-CLEB showed no significant difference in body weight to CONT. But the muscle wet weights of the HEART, SOL and EDL muscles increased in (+)-S-CLEB (HEART: +28%, SOL: +25%, EDL: +28%) and (-)-R-CLEB (HEART: +27%, SOL: +29%, EDL: +35%) compared to CONT. The wet weight of the HEART (1091.0 ± 69.9 mg, \( P < 0.05 \)) and EDL (210.7 ± 12.1 mg, \( P < 0.05 \)) in (-)-R-CLEB was significantly higher than CONT. Also, the ratio of muscle wet weight to body weight of the HEART, SOL and EDL muscles significantly increased in (+)-S-CLEB (HEART: +32%, \( P < 0.05 \), SOL: +29%, \( P < 0.01 \), EDL: +32%, \( P < 0.01 \)) and (-)-R-CLEB (HEART: +28%, \( P < 0.05 \), SOL: +31%, \( P < 0.05 \), EDL: +37%, \( P < 0.05 \)) compared to CONT. These results suggest a strong anabolic effect of CB enantiomers on striated muscles.

**Bone analyses.** The bone data including length and BMD are shown in Table 2. The length of the femur was not significantly different among the groups (CONT: 32.2 ± 0.57 mm, (+)-S-CLEB: 32.8 ± 0.45 mm, (-)-R-CLEB: 32.9 ± 0.71 mm). However, concerning the length of the tibiae among the three groups (CONT: 39.3 ± 0.43 mm, (+)-S-CLEB: 38.9 ± 0.26 mm, (-)-R-CLEB: 39.1 ± 0.90 mm), the (+)-S-CLEB (-1.2%) tibia was significantly shorter than CONT (\( P < 0.05 \)). Also, concerning the BMD of the femur among the three groups (CONT: 129.2 ± 5.2 mg/cm\(^2\), (+)-S-CLEB: 123.0 ± 3.1 mg/cm\(^2\), (-)-R-CLEB: 119.7 ± 3.0 mg/cm\(^2\)), BMD of (+)-S-CLEB and (-)-R-CLEB was significantly lower than CONT ((+)-S-CLEB: -5.8%, \( P < 0.05 \), (-)-R-CLEB: -8.2%, \( P < 0.01 \)). On the other hand, concerning the BMD of tibia among the three groups (CONT: 107.0 ± 5.0 mg/cm\(^2\), (+)-S-CLEB: 104.5 ± 1.6 mg/cm\(^2\), (-)-R-CLEB: 101.8 ± 4.0 mg/cm\(^2\)), there was no significant difference (see Table 2).

**Discussion**

The present study examined the hypothesis that (+)-S- and (-)-R-CB enantiomers would act differently on striated muscles and bones of young animals. In general, the side effects of drugs might be induced by the (+)-S-CB enantiomer\(^7\) or by the misuse of drugs. In Fig. 2, the initial rapid decrease in body weight may be due to anorexia described by Benson et al.\(^20\) Judging from the change of body weight in Fig. 2, the stimulation of (+)-S-CB to hypothalamic β-adrenergic receptor might be weaker than (-)-R-CB. In Table 1, we could not show the clear effect on body weight with (+)-S and (-)-R isomers of CB for 2 weeks. It was the same for the report of Sato et al.\(^31\) and Benson et al.\(^31\), but (+)-S-CLEB tended to be inhibited growth (see Fig. 2). In the previous report\(^3\), we showed inhibited growth with rac-CB administration for
Fig. 2  Effect of 2 weeks treatment of clenbuterol enantiomers on body weight of male rats.

Body weights of rats in three groups are shown. The filled diamond shows CONT (n = 6), the filled rectangle is (+)-S-CLEB (n = 6) and the filled triangle is (-)-R-CLEB (n = 6). At 8 weeks old, the (+)-S-CLEB, (-)-R-CLEB and CONT groups started the daily injection of (+)-S-clenbuterol, (-)-R-clenbuterol or equal volume of normal saline for 2 weeks, respectively.

Values are means ± SD. Significant difference between CONT and (+)-S-CLEB, *p < 0.05, **p < 0.01. Significant difference between CONT and (-)-R-CLEB, †p < 0.05, ††p < 0.01. Significant difference between (+)-S-CLEB and (-)-S-CLEB, #p < 0.05, ##p < 0.01.

Table 1.  Body weight and muscle mass after treatment of drugs for 2 weeks.

<table>
<thead>
<tr>
<th></th>
<th>CONT (n = 6)</th>
<th>(+)-S-CLEB (n = 6)</th>
<th>(-)-R-CLEB (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>365.5 ± 12.7</td>
<td>354.3 ± 14.1</td>
<td>361.3 ± 6.50</td>
</tr>
<tr>
<td>HEART</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wet weight (mg)</td>
<td>861.8 ± 40.4</td>
<td>1101.0 ± 95.8</td>
<td>1091.0 ± 69.9†</td>
</tr>
<tr>
<td>ratio (mg/g)</td>
<td>2.36 ± 0.16</td>
<td>3.11 ± 0.25*</td>
<td>3.01 ± 1.45†</td>
</tr>
<tr>
<td>SOL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wet weight (mg)</td>
<td>133.8 ± 11.7</td>
<td>167.5 ± 5.67</td>
<td>173.1 ± 15.1</td>
</tr>
<tr>
<td>ratio (mg/g)</td>
<td>0.366 ± 0.031</td>
<td>0.473 ± 0.01**</td>
<td>0.479 ± 0.04†</td>
</tr>
<tr>
<td>EDL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wet weight (mg)</td>
<td>155.7 ± 7.62</td>
<td>198.8 ± 10.8*</td>
<td>210.7 ± 12.1†</td>
</tr>
<tr>
<td>ratio (mg/g)</td>
<td>0.426 ± 0.020</td>
<td>0.561 ± 0.02**</td>
<td>0.583 ± 0.03††</td>
</tr>
</tbody>
</table>

Values are means ± SD.

Significant difference CONT and (+)-S-CLEB, **p < 0.01, *p < 0.05

Significant difference CONT and (-)-R-CLEB, ††p < 0.01, †p < 0.05
4 weeks. This might be partially explained by the adverse effect of (+)-S-CB. Furthermore, these effects might be related to a slightly shorter plasma terminal half-life of (+)-S-CB than (-)-R-CB, and a higher concentration of (+)-S-CB in the liver and kidney. CB is well known as a lipolytic agent. In the present study, the change of fat mass or liver, which is closely related to lipolysis and energy metabolism, was not examined; but von Deutsch et al. and Smith reported that (+)-S-CB remains in higher concentration than (-)-R-CB in the liver. The liver weight of rats was negatively affected by a high dose (2.0 mg/kg daily) of CB drinking for 4 days. Therefore, the change in body weight should be considered as the summation of various organs affected by CB.

In the present study, the heart and skeletal muscles of the SOL and EDL showed increased weights (see Table 2) like in previous reports. Cubria et al. reported cardiac hypertrophy by CB in mice and Duncan et al. showed undesirable effects by not only cardiac hypertrophy, but also increased collagen infiltration around blood vessels and in the wall of the left ventricle. Wet weight of EDL in (-)-R-CLEB (1.35 fold vs. CONT) was higher than (+)-S-CLEB (1.28 fold vs. CONT). Furthermore, that of SOL in (-)-R-CLEB (1.29 fold vs. CONT) was higher than (+)-S-CLEB (1.25 fold vs. CONT). EDL showed significantly higher anabolic effects in both (+)-S-CLEB (198.8 ± 10.8 mg, P < 0.05) and (-)-R-CLEB (210.7 ± 12.1 mg, P < 0.05) than CONT, but SOL showed that only the ratio of wet weight/body weight significantly increased in both CB groups. There was no clear difference between (+)-S-CLEB and (-)-R-CLEB. It suggests that both CB enantiomers were actively anabolic to striated muscle without much difference. These results were almost the same as the data of von Deutsch et al. using high dose rac-CB (1 mg/kg) despite an age difference. The anabolic effect on muscles might depend on various mechanisms of hypertrophy like the balance of protein synthesis and proteolysis, and might be different from the effects on bone. There were some contradictory reports that CB induced increased body weight, and no change in heart and skeletal muscles of animals. The reason might be due to the difference in type of animals, sex, age, administration method, dose, duration, etc. Unfortunately, in this study, we had no data on the difference in amount of physical activity or the amount of dietary intake among the 3 groups. We could speculate that a small difference would exist owing to the different effect on the hypothalamic β-adrenergic receptor related to the leptin content. Furthermore, our unpublished data using mRNA of MyoD and PGC-1α suggested that these CBs might participate differently in shift of a muscular fiber type. This might be related to the physical performance.

In this study, Table 2 showed a significantly shorter length of the tibia in (+)-S-CLEB (P < 0.05) than CONT and lower BMD of femur in both (+)-S-CLEB (P < 0.05) and (-)-R-CLEB (P < 0.01) than CONT. These results are partially supported by our previous study showing that the growth of both the femur and tibia were inhibited by rac-CB. (-)-R-CB might be more adversely active towards the BMD. (+)-S-CB acted negatively regarding the length of the tibia and BMD of femur. Such suggests the determination factors of bone length and BMD are various and different. Bonnet et al. also reported a shorter femoral length and reduced BMD as a deleterious effect of CB owing to the leptin-mediated mechanism via the hypothalamus. A leptin change may explain the inhibited growth of bones in our data according to its effect on the growth plate as described by Kishida et al. Hirosawa et al. suggested that (+)-S-CB clearance is smaller, and (+)-S-CB tissue distribution is larger than that of (-)-R-CB; and (+)-S-CB may cause adverse effects such as a reduction in blood pressure, increase in blood glucose level and elevation of glucocorticoid level. Glucocorticoid is one of the candidates for triggers for osteoporosis and a growth plate inhibitor. But in the present study we have no data about such. Cavalie et al. reported that CB decreased femoral length, diameter, and BMD of male rats, and it had no effect on osteocalcin, which is an active marker of osteoblast in plasma, but increased urinary de-

### Table 2. Effect of 2 weeks clenbuterol treatment on bone length and bone mineral density of male rats.

<table>
<thead>
<tr>
<th></th>
<th>CONT (n=6)</th>
<th>(+)-S-CLEB (n=6)</th>
<th>(-)-R-CLEB (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Femur</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone length (mm)</td>
<td>32.2 ± 0.57</td>
<td>32.8 ± 0.45*</td>
<td>32.9 ± 0.71</td>
</tr>
<tr>
<td>BMD (mg/cm²)</td>
<td>129.2 ± 5.2</td>
<td>123.0 ± 3.1*</td>
<td>119.7 ± 3.0 ††</td>
</tr>
<tr>
<td>Tibia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone length (mm)</td>
<td>39.3 ± 0.43</td>
<td>38.9 ± 0.26*</td>
<td>39.1 ± 0.90</td>
</tr>
<tr>
<td>BMD (mg/cm²)</td>
<td>107.0 ± 5.0</td>
<td>104.5 ± 1.6</td>
<td>101.8 ± 4.0</td>
</tr>
</tbody>
</table>

Values are means ± S.D.
Significant difference CONT and (+)-S-CLEB, **P < 0.01, *P < 0.05
Significant difference CONT and (-)-R-CLEB, ††P < 0.01, †P < 0.05
oxyypyridinoline, which is a resorption marker. Therefore, bone resorption might be increased by CB. It may explain our decreased BMD data (see Table 2) in part. This experiment showed the different results from the report by von Deutsch et al. using mature rats. They reported that treatment with 1.0 mg/kg rac-CB significantly increased tibia mass (115.4%), but had no significant effect on the femur. In contrast, treatment with 0.4 mg/kg rac-CB or either enantiomer (0.2 mg/kg each) had no significant effect on bone mass. In this experiment, we used higher purified (+)-S-CB and (-)-R-CB. Therefore, a high dose of CB enantiomer (2 mg/kg each) might be considered rather than rac-CB (1 mg/kg (+)-S-CB and 1 mg/kg (-)-R-CB) that was used in our previous study. The higher dose might induce a different result in part. Furthermore, in this experiment the stereochernical purities of the (-)-R and (+)-S enantiomers were 99.7% and 98.0%, respectively (see Fig. 1). The dose of contaminated (-)-R-CB in (+)-S-CB was calculated to be 0.04mg/kg. We could not eliminate the effect of contamination of (-)-R-CB on (+)-S-CB. Therefore, purified chemicals are needed to ascertain the effects of the drugs. But some of our data using (+)-S-CB was supported by low dose (0.2 mg/kg) reports of male SD rats showing no effect in heart weight and bones except EDL and SOL, but not supported by another report (0.01 mg/kg) using male Wistar rats. The difference might be due to the different strain of rats. Furthermore, three polymorphisms of the β-adrenergic receptor may be associated with BMD, so we need to examine the relationship between the CB enantiomers and these polymorphisms.

Described above, the use of CB is prohibited in sports globally, except in cases of clinical therapeutic use; but it is still detected as a food contaminant even in athletes. The function of this drug is very unusual and attractive in its preventive properties regarding some diseases like sarcopenia, Parkinson’s disease, cognitive deficits, and osteoporosis after space flight. However, more research is needed to avoid the deleterious side effects of clenbuterol enantiomers on various organs for human growth.

Conclusion

We examined the effects of two CB enantiomers, (+)-S-CB and (-)-R-CB, on the growth of striated muscles and bone in young male rats. Both (+)-S-CB and (-)-R-CB induced hypertrophied striated muscles (heart, SOL, and EDL). Regarding skeletal effects, (+)-S-CB induced a decreased length of the tibia and decreased BMD of femur, and (-)-R-CB induced decreased BMD of femur. These results show that (+)-S-CB and (-)-R-CB may function differently in part. Our hypothesis that (+)-S-CB has only an undesirable side effect on muscles and bones was shown to be incorrect. It is necessary to carry out more experiments with highly purified drugs to be able to learn the specific adaptation mechanisms, and be able to use such drugs effectively in the treatment of disease without adverse side effects.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

Acknowledgments

We are grateful to Ken-ichi Miyamoto for his gifts of enantiomers and his laboratory members for their technical assistance in the experiments. This work was supported in part by Grant-in-Aid for Scientific Research (No. 21500628 to T.K.) from the Ministry of Education, Science, Sports, and Culture of Japan, and the grant to T.K. in 2010 from the Shibuya Foundation for Science, Culture and Sports.

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